Growth factor dependent regulation of p70S6 kinase: Evaluation of domain specific signals sensitive to rapamycin

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Abstract

Phosphorylation dependent regulation of S6K1 has been proposed in accordance with the strategy of activation adapted by AGC family kinases. Accordingly three phosphorylations at the turn motif, HM and AL are considered critical for S6K1 regulation , while the turn motif phosphorylation is thought to be constitutive , the burden of the regulation is more or less entirely shared by the phosphorylations turn over at HM and AL of the enzyme. Although both phosphorylations are equally important without a question their individual roles in contributing to activation and mediating rapamycin sensitivity remains controversial. Phosphorylations of the enzyme at its HM brought about by rapamycin sensitive mTOR kinase is thought to prime the enzyme for PDK1 dependent phosphorylations at AL, in accordance with the prevalent dogma of sequential phosphorylations. Accordingly inactivation of mTOR by rapamycin resulting in loss of HM phosphorylation and resultant loss of phosphorylation at the AL is the model put forward to explain inhibition by rapamycin. The attribution of mTOR as the kinase responsible to engender HM phosphorylation, is however, based on scanty evidence carried forward simply on the basis of the ability of mTOR to bind rapamycin. In fact S6K1 mutants supposedly incapacitated for TOR kinase recruitment continue to be phosphorylated at the HM to suggest that effects attributed to TOS motif deletion may simply be conformational and not attributable to the loss of TOR kinase input at the HM. Further the implication of kinases other than TOR in mediating HM phosphorylation compound the ambiguity associated with TOR kinase involvement in mediating rapamycin inhibition. This study therefore, attempts to understand as to how the dynamics of the two phosphorylations in general and TOR mediated phosphorylation in particular relates to rapamycin mediated inhibition of S6K1.

We present evidence that baculovirus mediated expression of S6K1 in insect cells results in the failure of the enzyme to engender both HM and AL phosphorylations. The enzyme surprisingly continues to be inhibited by rapamycin. Evidence indicating redundant role of phosphorylation in general and TOR mediated phosphorylation in particular for S6K1 inhibition by rapamycin challenge the prevailing dogma. We also

provide evidence about the interdependent turnover of the two phosphorylations such as to exhibit all or none characteristics. The evidence that both AL and HM kinases are completely insensitive to rapamycin, suggests that their co-ordinate loss to rapamycin cannot be mechanistic and therefore has to be consequential. We finally show that the loss of the two phosphorylations is also associated with inhibition of S6K1 by agents like bupivacaine and lidocaine that target signalling pathways other than TOR, to strongly suggest that the loss of HM and AL phosphorylations is a general feature of S6K1 inhibition not necessarily governed by TOR inactivation.

Introduction, Background and Rationale

Mitogen signalling leads to coordinate activation of a number of pathways resulting in cell growth and proliferation (Dufner and Thomas, 1999, Martin and Blenis, 2002). One of the kinase families which respond to nutrient and growth factor signals is ribosomal protein S6 kinase (S6K). S6K was initially characterized as a kinase activity responsible for phosphorylation of ribosomal protein S6 in Swiss mouse fibroblasts (3T3 cells) (Edelmann et al., 1996). It was found initially that S6 protein phosphorylation is induced in response to growth factor stimulation which enhances protein synthesis in various cell types and accordingly several groups tried to identify the physiological kinase/s responsible for this S6 phosphorylation (Avruch et al., 2001, Dufner and Thomas, 1999, Mukhopadhyay et al., 1992). Two potential kinases identified were p70S6K (apparent molecular weight of 70 KDa) and p90-RSK (Jeno et al., 1988, Shima et al., 1998, Richards et al., 1999). Recent data suggests that that the former is the actual *in vivo* kinase responsible for S6 protein phosphorylations which are sensitive to the effects of immunosuppressant rapamycin (Price et al., 1992, Stewart et al., 1996).

1.1 S6K family of proteins

S6Ks (S6K1 and S6K2) are members of AGC family of Serine/Threonine kinases with S6K1 having two isoforms (p85 and p70) which are produced from same transcript using alternate translation start sites. The larger isoform being nuclear (p85 has 23 amino acid N-terminal NLS) and smaller one cytoplasmic (p70 S6 kinase). S6K2 in contrast to S6K1 has two nuclear isoforms (p54S6K and p56S6K), the longer isoform (p56S6K2) has an extra 13 amino acid amino-terminal sequence that serves as putative nuclear localization signal and the shorter isoform (p54S6K2) bears a carboxy terminal nuclear localization signal, conforming nuclear identity of both the kinases (Koh et al., 1999, Reinhard et al., 1992, Coffer and Woodgett, 1994).

Both S6K1 and S6K2 are functionally divided into distinct domains viz amino-terminal domain, catalytic domain, linker domain and carboxy-terminal domain (Grove et al., 1991). Although both the kinases share high degree of evolutionary

conserved sequence homology with catalytic domain being 83% homologous and

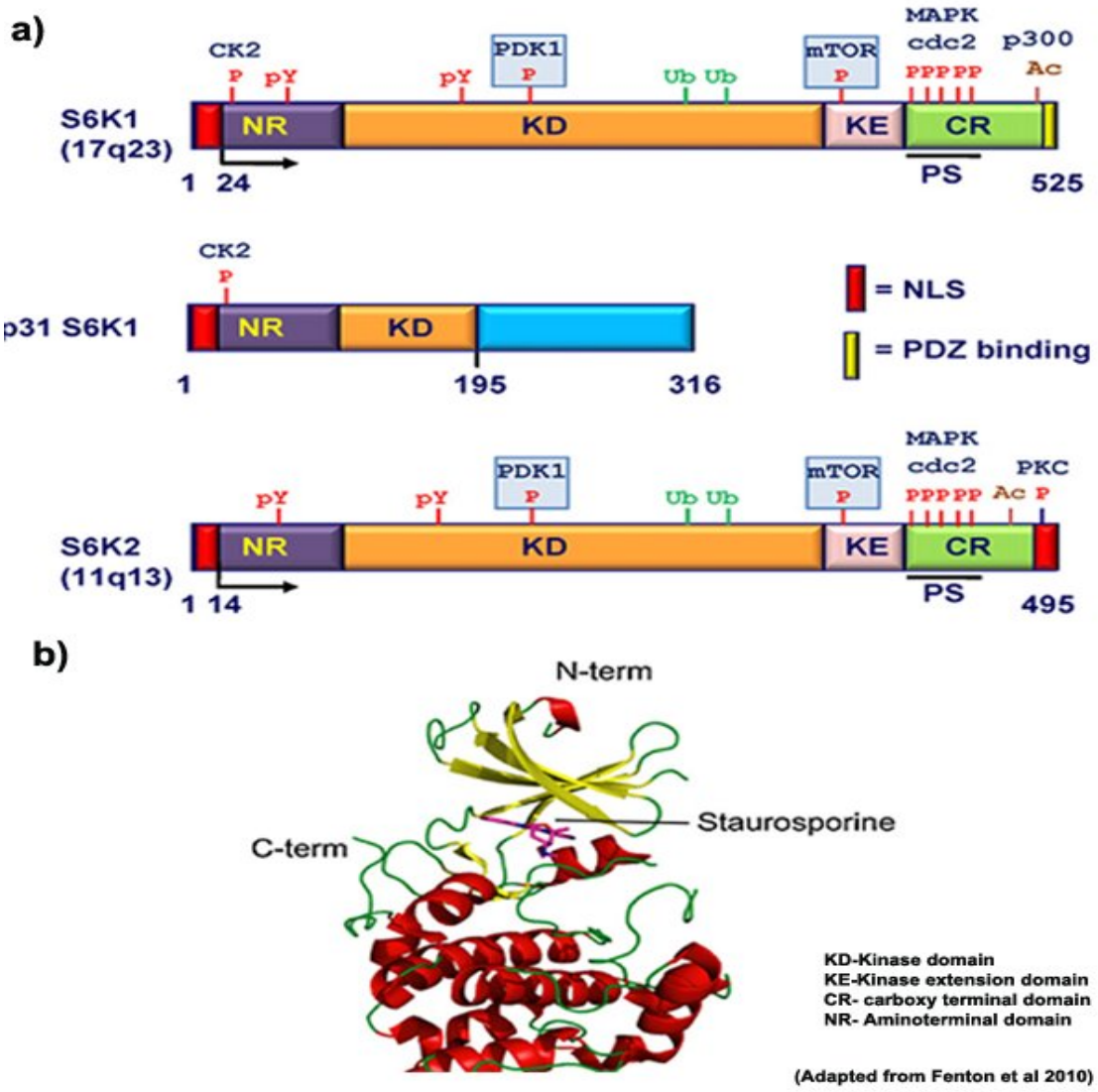


Figure 1.1: S6K 3D structure and domain organization with potential phosphorylating kinases along with their sites of action along with sites of other posttranslational modifications (a) Chromosome location of S6K isoforms in human and posttranslational modifications are depicted. The use of alternate translational start sites to produce the shorter isoforms of S6K1 (p70 S6K1) and S6K2 (p54 S6K2) are shown by arrows beginning at methionine 24 and 14 in S6K1 and S6K2 respectively. Regions containing nuclear localizations sequences (NLS) are depicted in red all other domains labelled (**b)** Ribbon representation of the 3D structure of S6K1 bound to staurosporine as solved by Sunami *et al*. (2010).

amino and carboxy terminal domains differing considerably in their sequence suggesting differential regulation mechanism and substrate specificity of the two kinases (Fenton and Gout, 2011). Schematic pictorial representation of S6K family is shown below in **Figure 1.1**. It is thought that this sequence difference in the flanking domains of catalytic site allows them to reside different cellular compartments or interact with different molecular targets e.g. p70S6K with its unique carboxy terminal PDZ binding domain allowing it to bind synaptic protein neurabin, to maintain actin cytoskeleton and S6K2 has proline rich carboxy terminus for interaction with SH3 domain and WW repeat containing proteins (Burnett et al., 1998).

1.2 Rapamycin - Mechanism of S6K inhibition and sensitive phosphorylation sites in S6 kinase.

Rapamycin is a fungal macrolide that has potent immunosuppressive properties and is an FDA approved immunosuppressive agent (Latta, 2000). Several laboratory reports have suggested potential antitumor activity associated with its use and potent anti proliferative effects on solid tumours in NCI screening program has been demonstrated (Alexandre et al., 1999). Rapamycin has also been shown to inhibit several cancerous cell lines in culture especially rhabdomyosarcoma, osteosarcoma, pancreatic cancer, neuroblastoma and glioblastoma demonstrating its potential as an anti-cancer agent (Hidalgo and Rowinsky, 2000, Seufferlein and Rozengurt, 1996, Vignot et al., 2005). Cellular target of rapamycin is a nutrient sensor complex protein known as mTOR (mammalian target of rapamycin). Rapamycin works by binding to a cellular protein FKBP12 and this complex directly binds to the mTOR FKBP12-rapamycin-binding (FRB) domain of mTOR to allosterically inhibit the complex (Chung et al., 1992, Abraham and Wiederrecht, 1996). During acute treatment, rapamycin inhibits assembled mammalian TORC1 (mTORC1) but not assembled mTORC2. Although the mechanism by which rapamycin inhibits mTORC1 remains incompletely defined, it is thought that rapamycin weakens the interaction between mTOR and raptor (regulatory associated protein of mTOR), an mTORC1 regulatory

partner thereby reducing mTORC1 intrinsic kinase activity, thus carrying inhibition to its downstream effectors S6K1 and 4EBP1 (Eukaryotic initiation factor 4E binding protein) (Nojima et al., 2003). S6 kinase lies downstream of mTOR pathway inhibited by rapamycin and evidenced by loss of its ability to phosphorylate S6 protein. Search for the rapamycin-sensitive regulatory phosphorylation sites led to the identification of T252 in the activation loop, as well as T412 and S404 in the linker domain, which connects the auto inhibitory domain to the catalytic domain (Pearson et al., 1995) .

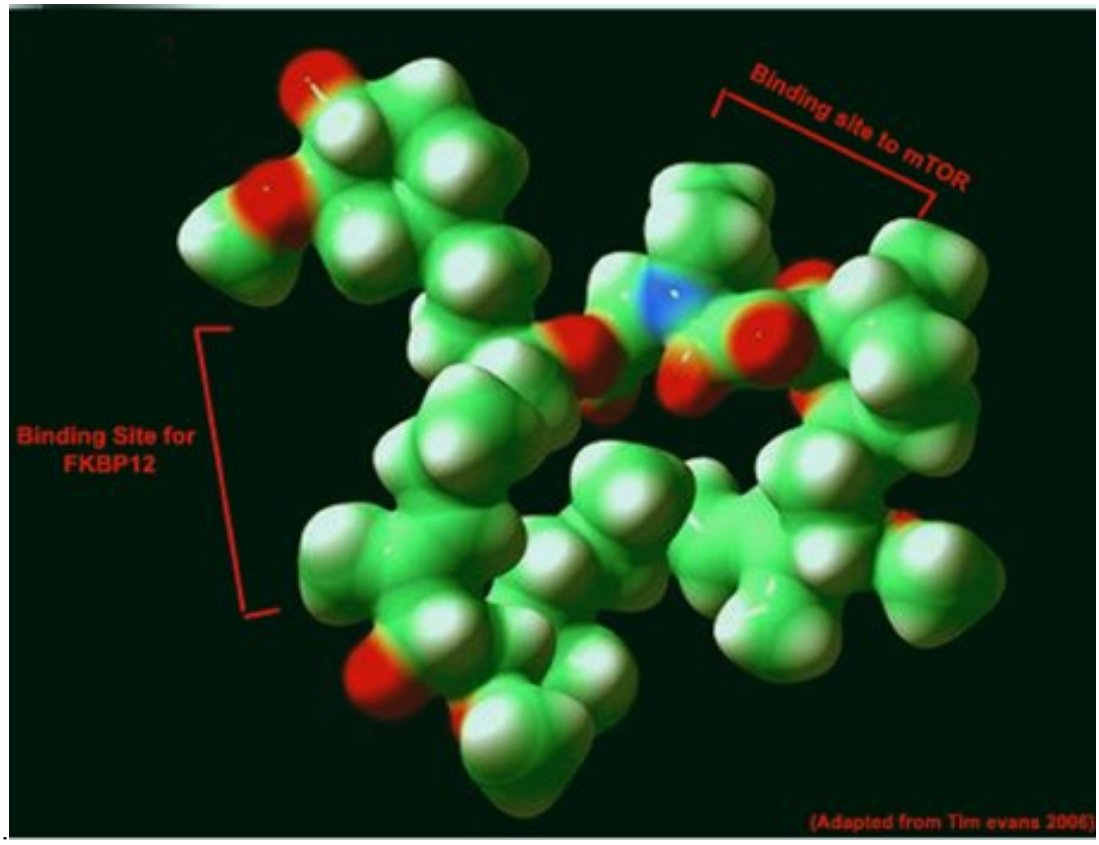


Figure 1.2: Electron-density model of a molecule of the immunosuppressant drug rapamycin, also known as sirolimus: It is mainly used to prevent rejection in organ transplantation, and is used in kidney transplants. It also has anti-fungal and anti-cancer properties, and was originally derived from soil bacteria found on Easter Island. The chemical formula is C51.H79.N.O13. The atoms are represented here as colour-coded blobs: carbon (green), hydrogen (white), nitrogen (blue) and oxygen (red) (Adapted from Dr. Tim Evans Science photo library)

Rapamycin exerts its inhibitory effect on S6K1 activation through either blocking the activation of an upstream S6K kinase or activating a phosphatase (Westphal et al., 1999). Additional studies showed that the rapamycin-sensitive sites were phosphorylated in response to mitogenic stimulation (Han et al., 1995) and that the three sites were dephosphorylated by rapamycin treatment in the hierarchical fashion T412 > S404 > T252, with T412 dephosphorylation most closely paralleling loss of kinase activity (Pearson et al., 1995).

A common characteristic of all three phosphorylation sites is that they are flanked by bulky aromatic amino acids, conversion of these sites to either acidic or neutral amino acids revealed that T252 and T412 were critical regulatory sites, whereas S404 appeared to play a modulatory function (Pearson et al., 1995). Mutational analysis also revealed that the principal site of rapamycin- induced S6K1 inactivation was T412, and substitution of an acidic residue in this position resulted in a kinase variant which had increased basal activity and was largely rapamycin resistant (Dennis et al., 1996, Pearson et al., 1995). Cooperation of the amino and carboxy termini in the activation of S6K1 was revealed by the finding that an amino-terminal truncated S6K was inactive (Weng et al., 1995b) not phosphorylated at the three rapamycin-sensitive sites in response to mitogens (Dennis et al., 1996). The kinase activity and phosphorylation were rescued by further deletion of the carboxy-terminal 104 amino acids, including the auto inhibitory domain generating a mutant termed S6K1 (DN54DC104). Surprisingly, this mutant was rapamycin-insensitive, but retained its sensitivity toward wortmannin. This observation combined with the finding that the carboxy- terminal truncated S6K1 retains rapamycin sensitivity raises the possibility that the rapamycin– FKBP12 complex enhances the activity of negative effectors, e.g., a phosphatase, which requires the amino terminus to exert its inhibitory effect. In contrast, the PI3K-dependent pathway appeared to be involved in regulating a positive element in the signalling pathway, as the S6K1DN54DC104 variant is still sensitive to wortmannin. Taken together, the above findings have led to a hierarchical model of kinase activation. The first step of kinase activation is mediated by phosphorylation of the S/T-P sites in the auto inhibitory domain. This step facilitates the next step,

phosphorylation of T412 in a PI3K-dependent manner, disrupting the interaction of the amino and carboxy termini of the kinase, thereby allowing phosphorylation of T252 and activation of the kinase in the final step (Dennis et al., 1998, Pullen et al., 1998).

1.3 Additional Regulatory Elements

Recent studies have revealed further elements of regulation which must still be considered before a complete picture of the mechanism of S6K1 activation is obtained. Consistent with the hierarchical model of kinase activation outlined above, substitution of the S/T-P auto inhibitory sites and T412 with acidic residues deregulates the phosphorylation of T252 in response to insulin *in vivo* (Dennis et al., 1998, Mukhopadhyay et al., 1992, Ferrari et al., 1992). Furthermore, T252 phosphorylation in this construct is unaffected by rapamycin & wortmannin treatment. However, kinase activity still responds significantly to insulin and is attenuated by wortmannin and rapamycin, indicating that S6K1 is regulated by an additional phosphorylation site. Preliminary studies indicate that this additional site corresponds to phosphorylation of S371 in the linker domain. Phosphorylation of S371 is increased by mitogen stimulation, and mutation of S371 to either an alanine or an acidic residue abolishes kinase activity (Moser et al., 1997). The role of S371, as well as that of S404, in the model of hierarchical phosphorylations which bring about kinase activation requires further study. A recent observation by another group has demonstrated phosphorylation at S371 to be constitutive in baculovirally expressed kinase in insect cells. Furthermore, they have shown phosphorylation at T252 to precede the one at hydrophobic motif (Keshwani et al., 2011). It seems likely that the mechanisms governing S6K1 and rapamycin sensitivity are independent of each other.

1.4 Upstream of S6 Kinase

Several kinases have been shown to phosphorylate this kinase at several sites and evidences of molecular cross talk exist without known roles of these interactions. An array of poorly defined post translational modifications of the kinase exist like lysine

acetylation and ubiquitination, however the biological significance of these modifications is yet to be established. Several of these kinases are discussed below:

1.4.1 mTOR (Mammalian target of rapamycin)

mTOR a 208kDa Ser/Thr protein kinase belongs to class IV PI3Ks. It is the one of the thoroughly studied kinase among PI3Ks due to its physiological significance and its relation with various pathological states (Schmelzle and Hall, 2000). Structurally divided into distinct functional domains , mTOR possesses an N-terminus containing up to 20 tandem HEAT motifs (including Huntington elongation factor 3 (EF3) a subunit of protein phosphatase 2A (PP2A) and TOR comprises the first 1200 amino acids), the C-terminus consists of mutated FRAP-ataxia-telangiectasia (FAT) domain, a transformation/transcription-domain-associated protein domain, an FRB domain (FKPB12 -FK506-binding protein 12 kDa-rapamycin binding), a catalytic kinase domain containing an ATP-binding site, a probable auto-inhibitory or repressor domain, and an FATC (FAT carboxy-terminal) domain (Bhaskar et al., 2009, Inoki et al., 2005).

The kinase domain is between the FRB domain (which is C-terminal to the FAT domain) and the FATC domain, located at the C-terminus of the protein. It is speculated that the HEAT repeats serve to mediate protein-protein interactions, the FRB domain is responsible to provide a docking site for the FKBP12/rapamycin complex, and FAT and FATC domains modulate mTOR kinase activity via unknown mechanisms. The catalytic kinase domain in the C-terminus has a high similarity to the catalytic domain of PI3K, so mTOR is considered a member of the PIKK (PI3K-related kinase) family, but there is no experimental evidence that it displays lipid kinase activity (Fingar et al., 2004, Schalm and Blenis, 2002).

mTOR exists in two distinct complexes - mTORC1 and mTORC2 depending upon the interaction partners and resultant sensitivity to a fungal macrolide rapamycin. Both the complexes are assembled as per the signalling input to mTOR. The mTORC1 complex is composed of the mTOR catalytic subunit, Raptor (regulatory associated protein of mTOR), PRAS40 (proline-rich Akt substrate 40 kDa) and the protein

mLST8/GβL. mTORC1 integrates four major regulatory inputs to its downstream effectors which include nutrients, growth factors, energy, and stress. The best characterized signalling pathway that regulates mTORC1 activity is the growth factor/PI3K/Akt pathway (Edinger and Thompson, 2002). PI3K/Akt signalling regulates mTORC1 through phosphorylation and subsequent inactivation of a negative regulator tuberous sclerosis complex (TSC) (Lee et al., 2007). TSC a hetero dimer composed of TSC1 and TSC2 subunits where TSC2 has GTPase activating protein (GAP) activity towards the Ras family small GTPase known as Rheb (Ras homolog enriched in brain) and TSC1/2 antagonizes the mTOR signalling pathway via stimulation of GTP hydrolysis of Rheb. TSC complex responses to changes in cellular energy levels through activation of AMP-activated kinase (AMPK), any cellular stress that depletes ATP viz oxidative stress, hypoxia, or nutrient deprivation leads to AMPK activation (Goncharova et al., 2002). Activated AMPK phosphorylates unique sites on TSC2, thereby activating the Rheb-GAP activity of TSC, which catalyzes the conversion of Rheb-GTP to Rheb-GDP and thus inhibits mTORC1 activity (Martin and Blenis, 2002, Inoki et al., 2003). Activation of mTORC1 results in phosphorylation and subsequent activation of its major downstream effectors 4EBP1 and S6K1 (Nojima et al., 2003), S6K1 activation is thought to regulate protein synthesis through phosphorylation of the 40S ribosomal subunit protein S6, which has been suggested to increase selective translational of a class of mRNA transcripts characterized by a 5-terminal poly-pyrimidine tract (Pende et al., 2004).

Recently, another mechanism supporting role of S6K1 in translation has been suggested via phosphorylation of eIF4B at Ser422, the mechanism which causes eIF4B to associate with eIF3 and promotes eIF4F complex formation (Raught et al., 2004). mTOR mediated phosphorylation of 4EBP1 also stimulates protein synthesis through the release of eIF4E from 4EBP1, allowing eIF4E to associate with eIF4G and other relevant factors to promote cap-dependent translation. Most recently, the growth factor receptor bound protein 10 (GRB10) was identified as an mTORC1 substrate which directly phosphorylates and simultaneously stabilizes GRB10, leading

to feedback inhibition of the PI3K pathway (Tari et al., 1999). Identification of the mTORC1-GRB10 interaction complements a negative feedback loop in which mTORC1 activation can inhibit the PI3K pathway through S6K1 mediated phosphorylation and degradation of insulin receptor substrate 1 (IRS1), and helps our understanding of the mechanism underlying mTORC1 inhibition of PI3K/Akt signalling.

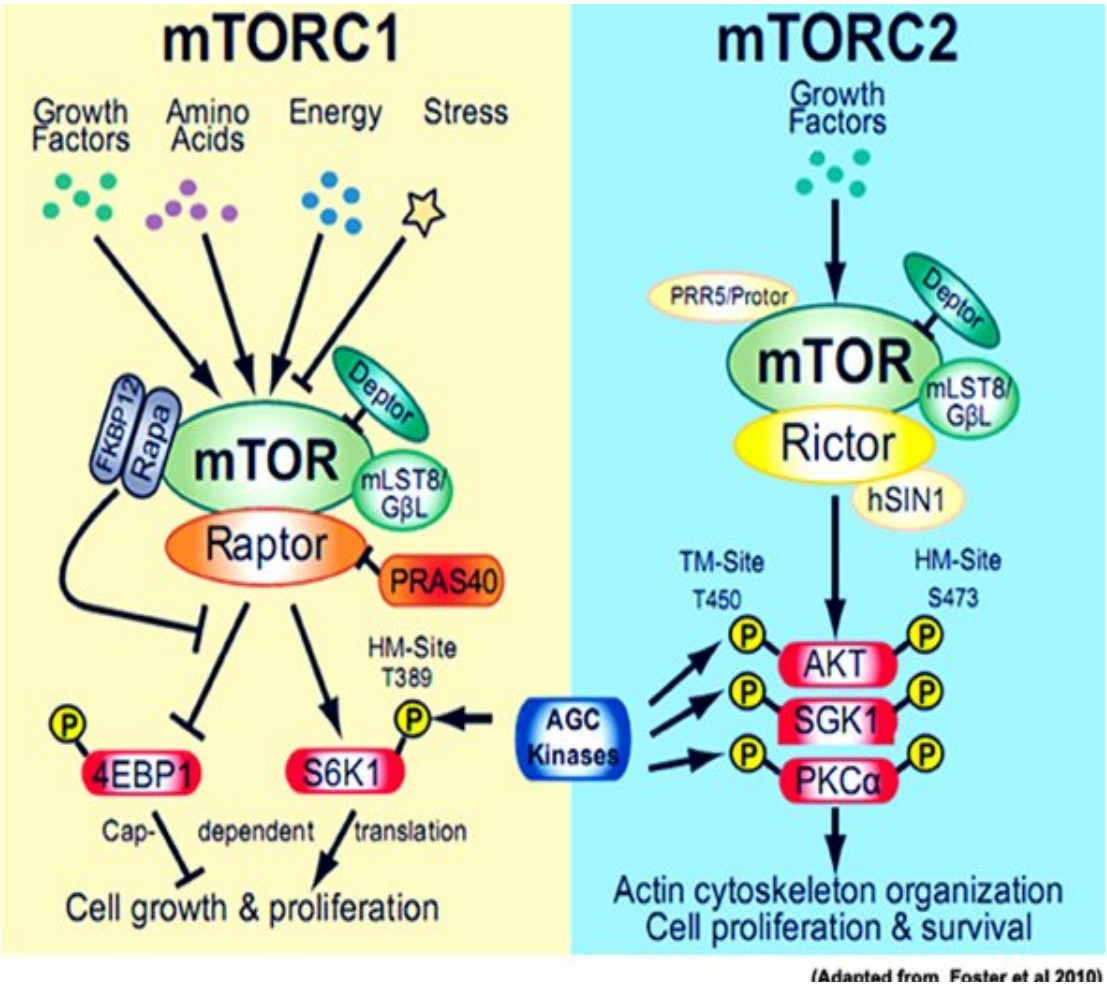


Figure 1.3: mTORC1 and mTOR C2 complexes: Distinct interaction partners and cellular functions and rapamycin sensitivity.

mTOR recognized as a central regulator of diverse array of vital cellular processes, including proliferation, growth, differentiation, and survival. The physiological importance of mTOR is undoubtedly demonstrated by the fact that the knockout of

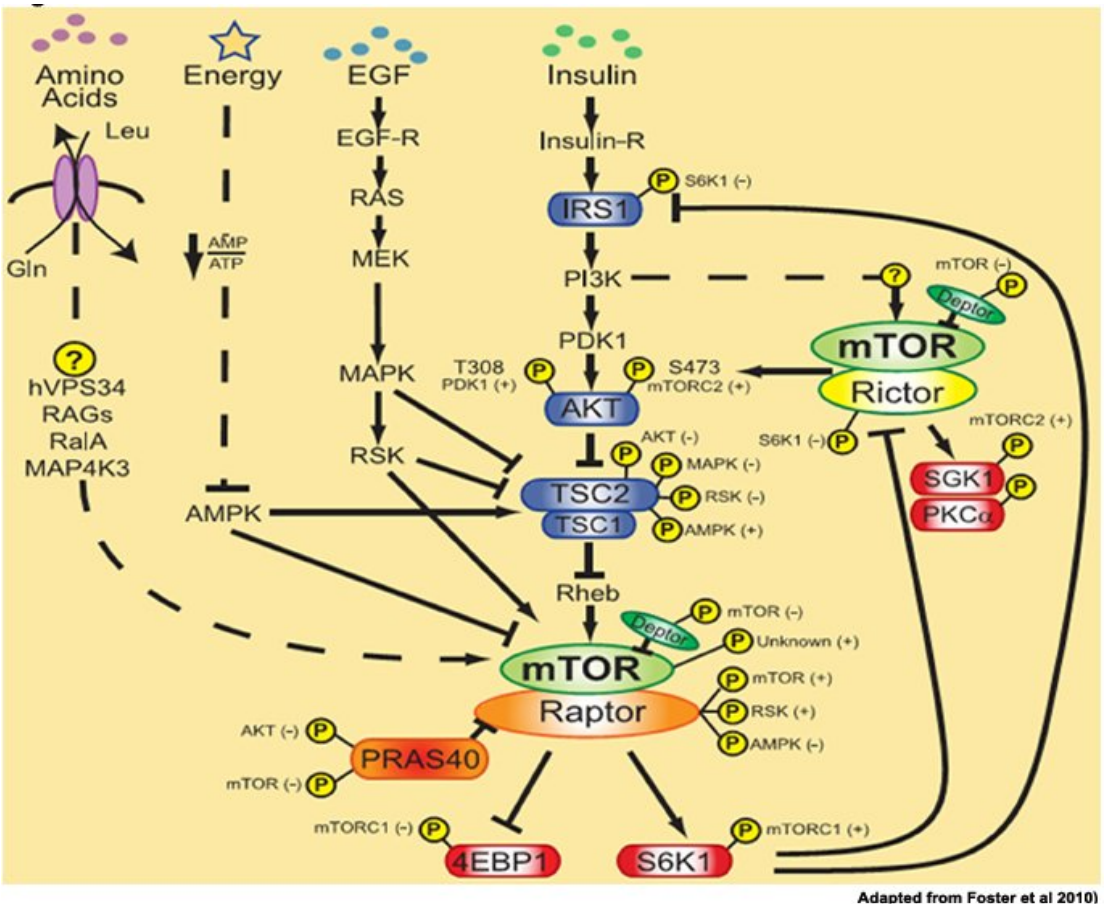


Figure 1.4: Regulation of mTORC and S6 kinase signaling networks. Growth factors/mitogens and nutrients promote mTORC1 signalling via phosphorylation mechanisms that converge on TSC and the mTORCs themselves. Insulin signals via its receptor (*Insulin-R*) to activate the PI3K/Akt/TSC/Rheb pathway; EGF signals via its receptor (*EGF-R*) to activate the Ras/MEK/MAPK/RSK pathway; amino acid sufficiency signals via hVps34 and the RAG and RalA GTPases; and energy sufficiency suppresses AMPK. Insulin/PI3K signalling likely promotes mTORC2 signalling via an unknown pathway. An mTORC1/S6K1-mediated negative feedback loop signals via two pathways to suppress PI3K/mTORC2/Akt signalling. *Arrows versus blocked lines* indicate activation or inhibition of protein function, respectively, by an upstream regulator. Phosphorylation events (denoted by *circled yellow P)* known to modulate protein function are shown. The kinases responsible for phosphorylation events are also indicated, with ( ) or ( ) denoting activation or inhibition of protein function, respectively.

mTOR in mice is primordially embryonic lethal, and the dysregulation of the mTOR pathway is associated with increased transformation and oncogenesis. Cumulative evidence demonstrates that mTOR plays a central role in the synthesis of key cellular proteins that are important for several aspects of cell growth and proliferation. Dysregulation of mTOR and other proteins in its signalling pathway often occurs in a variety of human tumours, and these tumour cells have shown higher susceptibility to inhibitors of mTOR than normal cells. Although Akt was believed originally to mediate mTOR Ser2448 phosphorylation, more recent work identifies S6K1 as the mTOR Ser2448 kinase. Prior to the identification of distinct mTOR complexes, mTOR Ser2481 autophosphorylation was believed to be insensitive to rapamycin and amino acid withdrawal, leading to the idea that modulation of mTOR intrinsic kinase activity does not universally underlie mTOR regulation. More recent work indicates, however, that rapamycin and amino acid withdrawal reduce mTORC1- but not mTORC2-associated mTOR Ser2481 autophosphorylation, consistent with the known sensitivities or lack thereof of mTORC1 and mTORC2 to these conditions. Thus, mTORC1- and mTORC2-associated mTOR Ser2481 autophosphorylation serves as a simple biomarker that monitors intrinsic mTOR catalytic activity. It is important to note that mTOR Ser2481 autophosphorylation was reported by another group to represent an mTORC2-specific event (due to undetectable Ser2481 autophosphorylation in mTORC1). These data may suggest higher stoichiometry of mTOR Ser2481 autophosphorylation in mTORC2 relative to mTORC1 (Foster and Fingar, 2010)

1.4.2 PDPK1 (Phosphoinositide dependent protein kinase 1)

PDPK1 was first identified as the activation loop kinase for protein kinase B (PKB) (Alessi et al., 1997b). These initial studies revealed that activation of PDPK1 was unaffected by stimuli that strongly activate PKB through PI3K. Instead, activation of PKB by PDPK1 required the binding of 3-phosphorylated phosphoinositides to the pleckstrin homology (PH) domain of kinases (Alessi et al., 1997b, Stephens et al., 1998). This served two purposes: to co-localize PDPK1 at the membrane with PKB

and to expose T308, allowing phosphorylation at this site by PDPK1 (Frodin et al., 2002). The fact that the sequence surrounding the activation loop of PKB was highly homologous to that of S6K1 and that PDPK1 appeared to be constitutively active suggested that PDPK1 may serve as the potential T252 kinase. Consistent with the hierarchical model of S6K1 activation , the S6K1 mutant with acidic residues at T412 and the four S/T P sites is a much better substrate for PDPK1-directed T252 phosphorylation *in vitro* and *in vivo* than wild-type S6K1 (Pullen et al., 1998), as these mutations probably expose the activation loop. Also consistent with earlier findings that T252 phosphorylation is largely rapamycin and wortmannin resistant in the mutant lacking the amino and carboxy termini (Dennis et al., 1996), the activity of PDPK1 toward S6K1 was found to be insensitive to pre treatment with either wortmannin or rapamycin (Pullen et al., 1998). Despite its high affinity for PtdIns (3, 4, 5) P3, PDPK1 is distributed equally in the cytosol and membranes, independent of any stimulus. This is consistent with S6K1 phosphorylation at T252, although S6K1 is not located at the membrane, and with the PtdIns (3, 4, 5) P3 independent nature of this response (Alessi et al., 1998, Pullen et al., 1998).

1.4.3 Akt/PKB

Although PDPK1-mediated S6K1 phosphorylation at T252 is not directly mediated by PtdIns (3, 4, 5) P3, it is dependent on T412 phosphorylation, which is a PI3K-dependent, wortmannin-sensitive step. As suggested by some workers, T412 phosphorylation is required to disrupt the interaction of the amino and carboxy termini to allow PDPK1 to phosphorylate T252. It has been suggested that PKB may directly or indirectly regulate T412 phosphorylation. This hypothesis was based on the observation that constitutively activated alleles of PKB targeted to the membrane induce S6K1 activation (Burgering and Coffer, 1995, Summers et al., 1996). However, the role of wild-type PKB in mediating S6K1 activation is controversial (Conus et al., 1998) , as demonstrated by the fact that the depletion of intracellular stores of Calcium has no effect on PKB activation, although it abolishes S6K1 activity. Conversely, they found that an increase in intracellular Ca results in full S6K1 activation, which is wortmannin-sensitive, but does not raise PKB activity.

These data imply that S6K activation can be achieved independently of PKB, requiring a separate calcium- dependent and wortmannin-sensitive input.

The importance of the studies of Conus *et al* have been recently underscored by experiments in which either activated alleles of PKB, differentially targeted to the membrane or cytoplasm, or a dominant interfering allele of PKB was co expressed with glycogen synthase 3 kinase (GSK-3), initiation factor 4E binding protein (4E-BP1) or S6K1 reporter constructs (Dufner et al., 1999). GSK-3 and 4E-BP1, as with S6K1, are downstream effectors of the PI3K signalling pathway and have been demonstrated to play a role in protein synthesis (Beretta et al., 1996, Clemens et al., 1996). GSK-3, through phosphorylation of the GTP–GDP exchange factor eIF2B, negatively regulates initiation factor eIF2-directed methionyl-tRNA binding to the 40S ribosome. In contrast, 4E-BP1 suppresses initiation factor 4E-directed mRNA binding to the 40S ribosome (Beretta et al., 1996) which is believed to play an important role in the translation of mRNAs with highly structured 5'untranslated regions. The results of co-expression studies showed that a constutively active wortmannin-resistant form of PKB, harbouring acidic residues at T308D and S473D (Akimoto et al., 1996), is sufficient to induce GSK-3 and 4E-BP1 phosphorylation, but not T412 phosphorylation and S6K1 activation (Edelmann et al., 1996). In parallel, a membrane-targeted dominant interfering PKB variant did not prevent insulin-induced S6K1 activation, whereas it blocked inactivation of GSK-3 and phosphorylation of 4E-BP1. The results strongly imply that constitutive membrane targeting of PKB may be misleading in assessing PKB function and that PKB resides on a parallel PI3K-dependent signalling pathway to that described for S6K1.

1.4.4 A typical PKCζ and PKCλ

The observation that the PI3K signalling pathway is important for S6K1 activation, but that PKB does not mediate this response, has placed attention on the atypical protein kinase C (PKC) family. Earlier studies showed that PKCz is regulated *in vitro* by PtdIns (3,4,5)P3 (Nakanishi et al., 1993) and that PKCl, as well as PKCz, is activated *in vivo* through a pathway involving PI3K (Akimoto et al., 1996) and

PDPK1 (Chou et al., 1998), but not PKB. Co-expression of a dominant interferingallele of PKCz antagonized S6K1 activation by epidermal growth factor (EGF), PDPK1, and activated alleles of either the small GTP binding protein Cdc42 or PI3K. A myristoylated constitutively active PKCz induced a modest activation of S6K1. In addition, they demonstrated in over expression studies that S6K1 forms complexes *in vivo* with PDPK1 and PKCz (Romanelli et al., 1999). Consistent with these findings, it was demonstrated that the amino-terminal region of S6K1, including the kinase domain, interacts with the kinase domain of PKCl, whereas the carboxy-terminal region of S6K1 is required for interaction with the regulatory domain of PKCl (Akimoto et al., 1998) . Akimoto *et al.* also reported that the regulatory domain of PKCl and a mutant without kinase activity antagonize serum-induced S6K activation, as well as serum-stimulated DNA synthesis and E2F activity in 293 cells. In contrast, dominant interfering alleles of S6K1 inhibited DNA synthesis and E2F activation. However, activated alleles of PKCl had no influence on basal or stimulated S6K activity, DNA synthesis, or E2F activity. These latter findings suggest that if the atypical PKCs are involved in S6K1 activation, they are necessary but not sufficient. It will now be important to determine whether the atypical PKCs are eliciting their effects through T412 phosphorylation. In this regard it has been recently claimed that phosphorylation of the corresponding site in PKCd, a member of the novel family of PKCs, is mediated by PKCz in a rapamycin-sensitive manner (Ziegler et al., 1999).

1.4.5 hVps 34

The mammalian homologue (hVps34) of Vps34p in association with another protein kinase Vps15p has been shown to play a role in multiple vesicular trafficking pathways.(Herman and Emr, 1990, Stack et al., 1993) Vps34 has also been implicated in autophagy in both yeasts and mammalian cells. Moreover, an hVps34-associated protein, beclin 1 is required for autophagy in mammalian cells (Liang et al., 1998, Yue et al., 2003). Although these data suggest that hVps34 is involved in nutrient-regulated pathways and a study by Byfield *et al* has hinted at the possibility that hVps34 may itself be regulated by the cellular nutritional state and hVps34 is required for insulin stimulation of S6K1. hVps34 is not regulated by insulin, nor does it affect

insulin-stimulated phosphorylation of Akt or TSC2. However, hVps34 is inhibited by amino acid or glucose starvation, and by activation of AMPK. These data suggest a novel role for hVps34 in nutrient sensing, and in the integration of signalling from amino acids and glucose to mTOR and S6K1. They have shown that S6K1 is regulated by that the Class III PI 3-kinase, hVps34 such that over expression of

hVps34 or the associated hVps15 kinase activates S6K1, and insulin stimulation of S6K1 is blocked by microinjection of inhibitory anti-hVps34 antibodies, over expression of a FYVE domain construct that sequesters the hVps34 product PI(3)P, or small interfering RNA-mediated knock-down of hVps34. hVps34 is not part of the insulin input to S6K1, as it is not stimulated by insulin, and inhibition of hVps34 has no effect on phosphorylation of Akt or TSC2 in insulin-stimulated cells. However, hVps34 is inhibited by amino acid or glucose starvation, suggesting that it lies on the nutrient-regulated pathway to S6K1. Consistent with this, hVps34 is also inhibited by activation of the AMP-activated kinase, which inhibits mTOR/S6K1 in glucose-starved cells. hVps34 appears to lie upstream of mTOR, as small interfering RNA knock-down of hVps34 inhibits the phosphorylation of another mTOR substrate, eIF4E-binding protein-1 (4EBP1) suggesting that hVps34 is a nutrient-regulated lipid kinase that integrates amino acid and glucose inputs to mTOR and S6K1 (Byfield et al., 2005).

1.4.6 DRAK 2 (Death associated receptor kinase)

A recent study by Mao et al suggested role of this serine /threonine kinase in regulating S6 kinase activity, it was seen that DRAK 2 phosphorylated S6K in an *in vitro* kinase assay and DRAK 2 expression positively regulated phosphorylation at the hydrophobic motif (T412) such that its over expression in NIT-1 cells leads to increased T412 phosphorylation and its down regulation using DRAK 2 specific siRNA produced the opposite effect. It is possible to link p70S6 kinase activation with islet apoptosis and that inhibition of p70S6 kinase phosphorylation by rapamycin contributes to the reduction of islet apoptosis after transplantation. This also suggests that inflammatory cytokines activate both the Drak2/p70S6 kinase and

mTORC1/p70S6 kinase pathways and that inhibiting one of them is only partially effective in reducing cell apoptosis. Indeed, when Drak2 up-regulation stimulated by cytokines was prevented by siRNA, islet apoptosis was decreased but was not totally prevented. Similarly, rapamycin only partially protected islet apoptosis from the cytokines. Dual inhibition of mTORC1 (with rapamycin) and Drak2 (with Drak2 inhibitors that are to be developed) might achieve better results in islet protection in terms of cytokine-induced cell apoptosis. Thus it seems probable that mTOR/S6K pathway runs parallel to DRAK2/ S6K pathway and molecular cross talk between these two pathways is circumstantial and prevalent under conditions involving cell apoptosis and other survival mechanisms.

1.5 Regulation of the kinase by intracellular Amino Acids

Essential amino acids inhibit autophagic proteolysis and are effective in stimulating S6 phosphorylation (Blommaart et al., 1995). Insulin potentiates the effect of amino acids while rapamycin completely abolishes this response. These findings led to the suggestion that phosphorylated S6 may mediate inhibition of autophagy, which is also employed in normal cell homeostasis to eliminate aged proteins and organelles (Dunn, 1994). Although S6 phosphorylation was proposed as a negative effector of autophagy, it is more probable that amino acid deprivation would trigger a negative signalling event which would suppress the biogenesis of translational machinery as reflected by S6 dephosphorylation. Consistent with this notion, amino acid deprivation blocked S6K1 activation as well as 4E-BP1 phosphorylation in CHO cells (Wang et al., 1998) and HEK293 cells (Hara et al., 1998). Conversely, re-addition of amino acids restores S6K1 activity and 4E-BP1 phosphorylation to levels induced by mitogens. More striking, a rapamycin-insensitive mutant of S6K1, lacking an amino and carboxy terminus, was not inactivated by amino acid deprivation, suggesting that this effect is mediated by mTOR (Hara et al., 1998). Terada and co-workers have provided evidence that deacetylated tRNA is the mediator of S6K1 inhibition by amino acid deprivation and have demonstrated that amino acids can restore S6K1 activation in amino acid-deprived and rapamycin-treated Rh30 cells constitutively expressing a rapamycin-resistant TOR variant (Iiboshi et al., 1999). Taken together,

these data indicate that mTOR is directly involved in regulating S6K1 activity induced by amino acids. It has been reported that constitutive membrane targeting of PKB promotes system amino acid transport which could provide a mechanism by which membrane-targeted alleles of PKB induce S6K1 activation. Thus, amino acids can act as direct initiators of signal transduction pathways.

1.6 S6K Functions and intracellular substrates

S6K pathway is activated in response to cell growth signals by coordinate influence of a number of pathways including mTOR, PI3-kinase and MAPK, through ordered phosphorylation events directed at multiple sites (Schmelzle and Hall, 2000). S6K is a multifaceted effector that regulates cell growth and proliferation by phosphorylating multiple ways. A few of the S6K regulated processes are briefly outlined below:

1.6.1 Cell growth

S6k was initially known as a kinase phosphorylating S6 protein of 40S ribosomal subunit at five C-terminal serine residues in a sequential manner S236 > S235 > S240 > S244 > S247 to regulate cell growth, mice lacking rps6 -/- show remarkably small phenotype as do the S6K -/- phenotypic variants in spite of having multiple kinases that phosphorylate S6 like p^{90}rsk in response to mitogens. An important event that was initially attributed to S6 phosphorylation is selective translation of mRNAs characterized by 5'- oligo Pyrimidine tract (5'-TOP) was later confirmed to be an independent event (Ruvinsky et al., 2005). This is a complex issue that needs to be focussed on, as it is not clear what physiological conditions cause kinase selective phosphorylation of rps6 and to what effect. The study of knock-in mice in which these five phosphorylation sites in rpS6 were replaced by alanine residues (rpS6) has provided much-needed insight into the physiological role of rpS6 phosphorylation *in vivo* (Ruvinsky et al., 2009, Ruvinsky et al., 2005). Although rpS6-/-mice are viable and fertile, they display a remarkable phenotypic overlap with S6K1-/- mice and strikingly, a cell growth defect is evident in both cases. In both rpS6P-/-and S6K1-/- mice, hypoinsulinaemia decreased cross-sectional area of the myotubes results in a reduction in size of the pancreatic cells leads to muscle weakness and cells isolated

from both genotypes display defective cell growth (Pende et al., 2000, Ruvinsky et al., 2009, Shima et al., 1998).

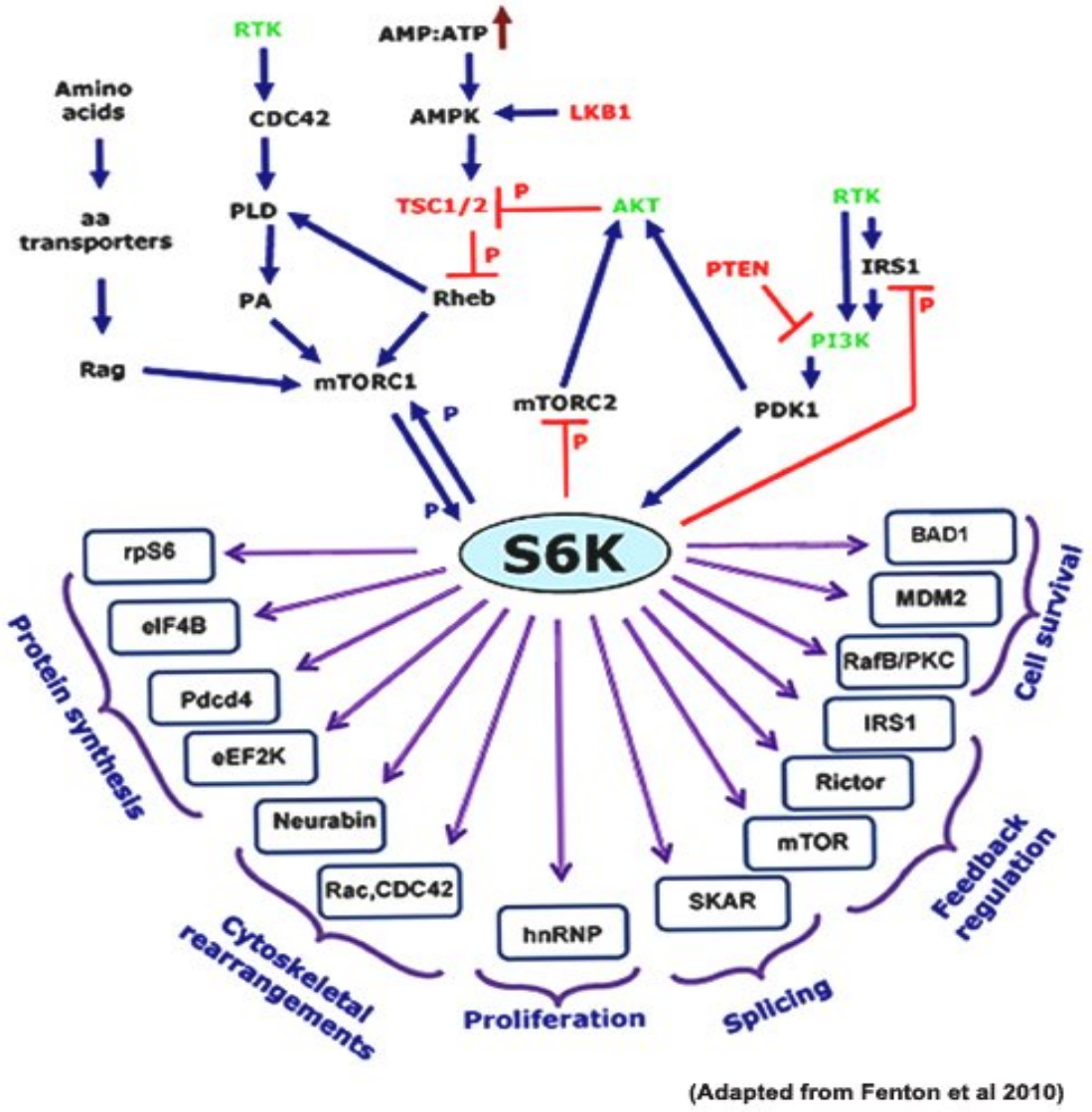


Figure 1.5: Key upstream regulators and downstream effectors of S6K signalling. Major components of the signalling pathways regulating S6K activation are shown, with proto-oncogenes in green and tumour suppressors in red. S6K downstream substrates are also shown along with their relation with other pathways (Fenton and Gout, 2011).

The cell growth phenotype common to these mouse models is particularly interesting given that S6K1- /- mice display minimal defects in rpS6 phosphorylation, while S6K2- /- mice grow to normal size despite a significant reduction in rpS6 phosphorylation (Pende et al., 2004, Shima et al., 1998). Selective recruitment of S6K1 and S6K2 into distinct protein complexes and sub cellular compartments suggests differential phosphorylation rpS6 or phosphorylation of rpS6 may be required at a specific developmental stage, at which S6K1 but not S6K2 is active in the affected cell lineages.

The link between S6K1, rpS6 phosphorylation and cell growth is clearly an area of great interest for further study. In addition to re-establishing a role for rpS6 phosphorylation in the control of cell growth, analysis of the rpS6P-/- mice also confirmed that translation of a specific subset of mRNAs containing an oligo pyrimidine tract at the 5' terminus (5TOP mRNAs), a process long thought to be under control of rpS6 phosphorylation occurs entirely independently of this event (Ruvinsky et al., 2005). The fact that 5/S6K1-/-/S6K2-TOP mRNA translation is unaffected in mice, which display minimal rpS6 phosphorylation, further demonstrates the lack of association between these events *in vivo* (Pende et al., 2004).

1.6.2 Cellular metastasis and S6 Kinase

S6 kinase has been implicated to regulate cellular metastasis in glioma cells wherein mTOR/S6K axis has been shown to be constitutively activated and using silencing RNAs against S6K partially rescues the transformed phenotype (Nakamura et al., 2008) in addition to its reported constitutive activation in oesophageal squamous cell carcinomas (ESCC) (Hou et al., 2007).

1.6.3 Translational control

- It regulates intracellular pool of eIF3 (pre-initiation complex component) in a serum sensitive manner through mTOR pathway (Holz et al., 2005).
- It regulates cap dependent translation by activating eIF4B at serine 422 under mTOR influence in response to nutrient supply (Raught et al., 2004).

- It also controls translational initiation by phosphorylating PDCD4 which is a negative regulator of eIF4A, marking it for degradation by ubiquitin ligase βTRCP (Dorrello et al., 2006).
- It phosphorylates and inactivates elongation factor 4- kinase to regulate cell growth (Wang et al., 2001b) .

1.6.4 Cell cycle progression

S6K1 has been related to G1 to S progression by a number of workers (Lane et al., 1993), however; a direct role in the process has not been established. A study has reported that phosphorylation of S6K1 aids in cell cycle progression thorough phosphorylation of oestrogen receptor leading to activation of its target genes resulting in proliferation (Yamnik et al., 2009). In this study, the proliferation of cell lines in which S6K1 was highly expressed due to amplification of 17q23 was sensitive to rapamycin, while those with lower S6K1 levels were relatively resistant, thus the contribution of S6K1 to proliferation may be apparent only at supra physiological expression levels. This could be advantageous in the context of targeting S6K in cancer, where the aim is to minimize effects on normal cells. S6K1 has been also shown to phosphorylate CREB (cAMP response element binding protein) and transcription factor UBF-1, which in turn activates RNA polymerase 1 driven transcription of ribosomal RNAs aiding in ribosomal biogenesis. Serum stimulation driven mTORC1 mediated activation of hNRPs especially hNRP-F by S6K2 aids in cell proliferation. Significantly however, cardiac hypertrophy, a response dependent upon ribosome biogenesis, is unaffected in S6K -/- mice, thus there is no absolute requirement for S6K function at either the transcriptional or translational levels of this process (McMullen et al., 2004).

1.6.5 Cell survival signalling

Pro-apoptotic molecule BAD, an Akt substrate has been shown to be phosphorylated by S6K1 at Ser136 causing its 14-3-3 dependent sequestration in cytoplasm and inactivating its function (Harada et al., 2001). S6K has been shown to phosphorylate P53 ubiquitin ligase MDM2 causing cell survival (Lai et al., 2010). S6K1 modulates

several actions of Mdm2, like 163/183 phosphorylation, nuclear cytoplasmic shuttling, and Mdm2-mediated ubiquitination of its substrates likely through interaction between these two proteins. S6K regulates the stability of p53 - the best-studied Mdm2 substrate, cell death response under genotoxic stress in normal cells. Thus, the mTOR-S6K pathway has a function in DNA damage response by transmitting pro-apoptotic signals and may also regulate tumorigenesis. Several initial studies have indicated that p38 MAPK can directly act on Cdc25 and p53; it was however later revealed that p38 MAPK can also regulate p53 stability through the mTOR-S6K Mdm2 pathway, thus highlighting the importance of p38 MAPK further in DNA damage response. Enriched environment will lead to S6K1 T412 phosphorylation and activation, enhanced S6K1-Mdm2 complex formation, and Mdm2 cytoplasmic retention, allowing maximal p53 induction upon genotoxic stress, which might be needed to counteract the strong mitogenic signals and enhanced protein synthesis mediated by the mTOR-S6K pathway. On the other hand, poor environment will downplay mTOR-S6K signalling, leading to reduced p53 induction upon genotoxic stress. This might be sufficient to cause cell-cycle arrest in the presence of weak mitogenic signals. Therefore, S6K1–Mdm2 interaction may provide the link between cells status (nutrients, energy, and growth factors) and their response to DNA damage. This study establishes that S6K1 is not only a kinase for Mdm2 S163 phosphorylation but also a physical interacting partner under genotoxic stress. S6K1 can phosphorylate Mdm2 on S163 *in vivo* and *in vitro* and is required for S163 phosphorylation in response to DNA damage through activation of mTOR-S6K1 axis mediated through p38a MAPK. Activation of S6K1 leads to a high titre complex formation with Mdm2 which in turn inhibits Mdm2-mediated p53 ubiquitination and promotes p53 induction. Deactivation of mTOR-S6K1 signalling leads to Mdm2 nuclear translocation, which is facilitated by S163 phosphorylation, a reduction in p53 induction, and an alteration in p53-dependent cell death. These findings thus establish mTOR-S6K1 as a novel regulator of p53 in DNA damage response. S6K1–Mdm2 interaction presents a route for cells to incorporate the metabolic/energy cues into

DNA damage response and links the aging-controlling Mdm2– p53 and mTOR-S6K pathways.

1.6.6 Cell migration: A function related to p70S6K activation

Coordinate assembly and rearrangement of cytoskeleton dictates movement of across a substratum through polymerization and depolymerisation of actin filaments leading to extension of the cytoskeletal structures at the leading edge and translocation of the cell through the forward motion of the cell at the leading edge and detachment at the trailing edge (Ip et al., 2011). These cellular structures are formed in response to actin polymerization and the formation of actin stress fibres, the basic component of the cytoskeleton necessary for focal contacts (adhesion), migration and maintaining cellular shape. At the rearward side of the cell are tails that anchor the cell to the substratum and that are released in a controlled manner as the cell migrates forward. Complex signalling pathways regulate formation of these cytoskeletal structures involving receptor activation mediated by signalling proteins and second messengers such as PI-3,4P2, phospholipase C, PKC, Ca^{2+}, PI 3-kinase, Rho and Rac GTPases and MAPK (Wells, 2000). Association of p70S6K with small GTPases like Rac1 and cdc42 which are known to regulate membrane ruffling, migration and actin polymerization has shown to mediate some of these effects (Chou et al., 2003). Dominant-negative Rac1 over expression in several cell types prevented growth factor-induced p70S6K activity, suggesting that Rac1 activates p70S6K. In addition, activation of p70S6K by expression of an activated allele of Rac1 is inhibited by rapamycin and the PI 3-kinase inhibitor wortmannin. The results of both experiments support a role for p70S6K in regulation of the cytoskeleton, except that rapamycin had no effect on membrane ruffling or stress fibre formation. Studies showing Rac1-mediated activation of p70S6K is unrelated to cytoskeleton reorganization have not been published. In contrast, several studies have shown a role for p70S6K in cytoskeleton regulation and cell migration. p70S6K has been shown to co-localize with actin stress fibres, suggesting that p70S6K activation plays a role in actin polymerization. Another observation, that thrombin stimulation causes a shape change effect that is characterized by elongation and organization of stress fibres and this

effect is inhibited by treatment with rapamycin (Crouch, 1997). Nitric oxide donors have been found to increase growth factor-stimulated p70S6K activity and this potentiation of p70S6K activity is associated with a prolonged shape change effect and enhancement of tails, both morphological features that may be enhanced in rapidly migrating cells (Berven et al., 1999). Based on several morphological and biochemical results, it has been proposed that p70S6K is involved in regulating the migration of 3T3 fibroblasts and thus present a potentially novel function of p70S6K. Clearly, p70S6K is important for regulation in translation. Thus, growth factor-induced translocation of p70S6K to the actin cytoskeleton and leading edge of the cell followed by localized synthesis of key protein regulators of filopodia or lamella extension is one possible mechanism. In support of this idea, it has been shown that p70S6K may be targeted to nerve endings via its interaction with Neurabin, an F-actin binding protein that is highly expressed in nerve tissue (Burnett et al., 1998). In this study, p70S6K and Neurabin were shown to co-localize in brain sections by *in situ* hybridization and were both enriched in the synaptosomal fraction in rat brain. Although localized protein translation in nerve terminals has not been established, it is possible that p70S6K functions to increase synthesis of proteins required for the assembly of actin cytoskeletal structures that are involved in neurite outgrowth or growth cone formation. Thus, it is possible that the function of p70S6K at the synapse may be analogous to its role in migration. Recently, it has been shown that mTOR interacts with gephyrin, a protein that is necessary for the clustering of glycine receptors at the postsynaptic terminals in spinal cord neurons. In these studies, expression of mTOR mutants that were unable to bind gephyrin failed to activate the downstream targets of mTOR, p70S6K and 4E-BP1. Furthermore, while mTOR expressed in HeLa cells appears uniformly distributed throughout the cytoplasm, co expression of mTOR with gephyrin causes aggregation of mTOR at polarized regions of the cell, suggesting that gephyrin may influence mTOR (and consequently p70S6K) signalling through its role in clustering receptors or other signalling molecules that contain a gephyrin-binding domain (Sabatini et al., 1999).

1.7 Regulation of S6 kinase by ubiquitination

Several studies have pointed at regulation of S6 kinase by ubiquitination and subsequent 26S proteosome mediated degradation, although the physiological conditions under which S6K degradation is triggered are currently unknown. S6 Kinase has been shown to interact with E3 ubiquitin ligase ROC 1 (regulator of cullins 1) (Panasyuk et al., 2008), and this interaction is responsible for ubiquitination of S6K1 as shown by using SiRNA against ROC1, which inhibit S6K ubiquitination and it's over expression produces the opposite effect. S6K has been shown to get ubiquitinated at multiple Lysine residues in the catalytic domain; the identity of these residues however remains to be identified. Furthermore, the finding that S6Ks are also acetylated and that treatment of cells with deacetylase inhibitors leads to a stabilization of S6K2 suggests that, as with other proteins such as p53, the ubiquitination and degradation of S6Ks may be opposed by lysine acetylation. Thus regulation of S6K by ubiquitination adds a further node of complexity to regulation mechanism of S6 Kinase (Gwalter et al., 2009).

1.8 Regulation of S6 kinase by Akt/MAPK/ERK

S6K1 acts downstream of MAPK/PI3 kinase/Akt signalling pathways to regulate cell cycle progression primarily due to its influence on cellular protein synthesis, the role of S6K1 also stands established in other cellular processes like autophagy, apoptosis and aging implicating a complex network of signalling events in its regulation. While mTOR is considered to be the major activating input, kinases that include AKT, GSK3β and others have also been implicated to influence S6K regulation. Interaction between MAPK and S6K1 suggestive of cross talk between pathways stands as well characterized (Wang et al., 2001a), such that inhibitors viz PD-98059 that inactivate MAPK/ERK pathway reproduce the response similar to S6K1 inhibition (Lehman and Gomez-Cambronero, 2002). However the functional implication of this process is not fully understood, it seems likely that MAPK pathway acts upstream of S6 kinase but the mechanism underlying this regulation is not fully understood.

Similarly Akt-a downstream effector of PI3- kinase pathway is a known activator of S6K1 that caters to the response associated with insulin signalling, inhibition of such signals is therefore likely to have a bearing on the activity status of the S6K1 enzyme (Dufner et al., 1999). Observations that expression of activated forms of Akt led to the activation of p70S6K implied Akt might mediate mitogenic signalling through activation of p70S6K (Riemenschneider et al., 2006). To clarify the relationship between signalling through these two kinases, we have examined their regulation by various mitogenic stimuli. In this study we have focused on the role of calcium in the regulation of each kinase in BalbC-3T3 fibroblasts. Depletion of intracellular calcium stores by EGTA pre-treatment has no effect on growth factor-induced Akt activation but completely abolishes p70S6K stimulation. Furthermore, although Akt is insufficient for the activation of p70S6K achieved independently of Akt to conclude that Akt and p70S6K lie on separate signaling pathways (Conus et al., 1998, Lizcano et al., 2003).

1.9 Baculovirus expression and insect cell system

Baculoviruses are the most prominent viruses known to infect insect population. They are double-stranded, circular, super coiled DNA molecules in a rod-shaped capsid. More than 500 baculovirus isolates (based on hosts of origin) have been identified, most of which originated in arthropods, particularly insects of the order Lepidoptera (Jehle et al., 2006). Two of the most common isolates used in foreign gene expression are Autographa californica multiple nuclear polyhedrosis virus (AcNPV) and Bombyx mori (silkworm) nuclear polyhedrosis virus (BmNPV) (Jones and Morikawa, 1996). AcNPV is usually propagated in cell lines derived from the fall armyworm *Spodoptera frugiperda* (Sf9 and Sf21 cells) or from the cabbage looper *Trichoplusia ni* (Marheineke et al., 1998). Cell lines are available that grow well in suspension cultures, allowing the production of recombinant proteins in large-scale bioreactors. Foreign genes are expressed in insect cells using baculoviral expression system (Kitts and Possee, 1993, Hartig and Cardon, 1992). Genes to be expressed are commonly placed under the control of strong AcNPV polyhedrin promoter replacing the cDNA

of polyhedrin gene which is expressed very late in the course of infection (Kang, 1988). Thus, recombinant product is expressed in place of the naturally occurring polyhedrin protein. In the late phase of infection, the virions are assembled and budded recombinant virions are released (Roy et al., 1997). Usually, the recombinant proteins are processed, modified, and targeted to the appropriate cellular locations. Various post translational modifications of mammalian proteins are made correctly comparable to their native location and therefore offer an added advantage to the higher expression status (Lenhard et al., 1996).

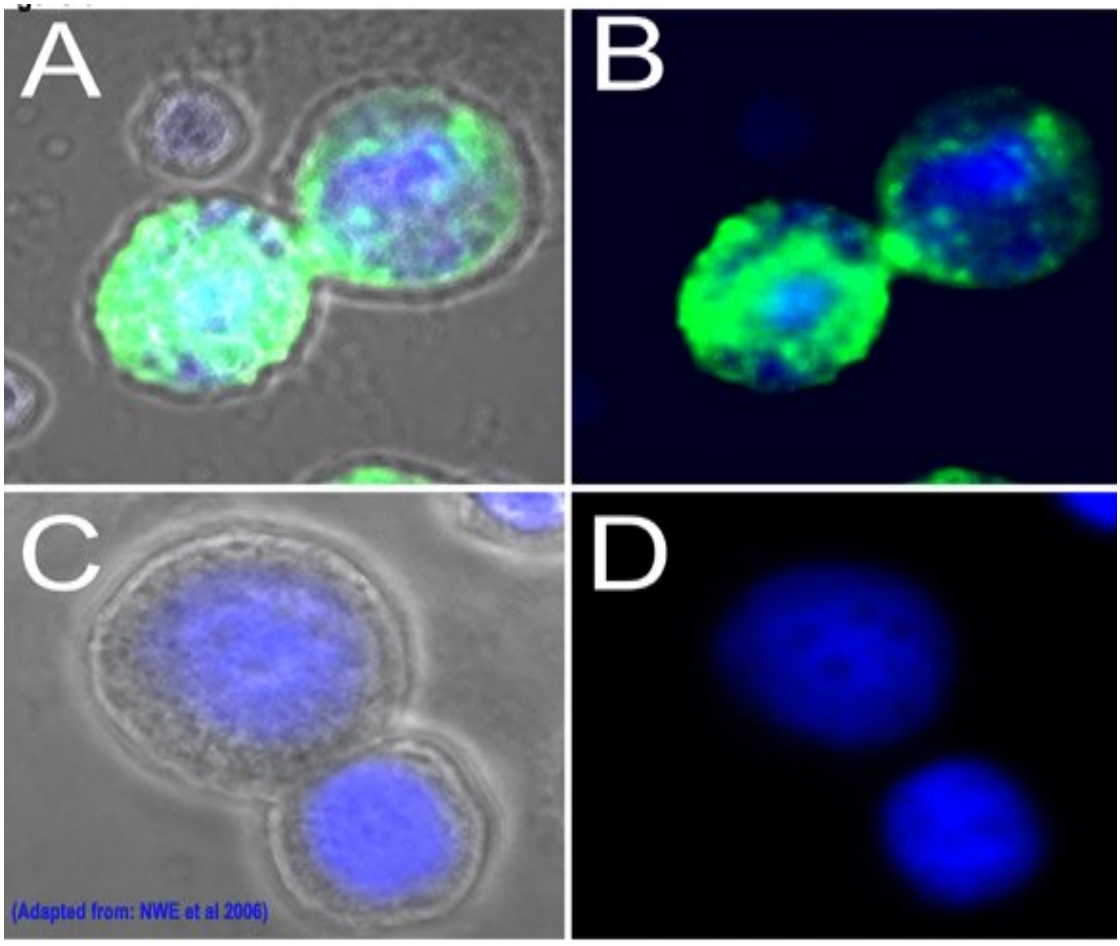


Figure 1.6: Confocal imaging of infected and uninfected insect cells: Recombinant baculovirus infected Sf9 cell (A and B) and uninfected Sf9 cell (C and D). DAPI was used to stain cell nuclei and monoclonal antibody specific to HA1 of avian influenza H5 was used to localize rHA1 in recombinant baculovirus infected cells and uninfected cells (Nwe et al., 2006).

As heterologus proteins are expressed very late in the course of infection, it is considered a system of choice for expression of toxic proteins, which are not possible to study in mammalian expression systems. Insertion of the foreign genes under AcNPV destroys a lethal deletion (Baculogold expression system), therefore only recombinants survive adding to the specificity of the system. Another advantage offered by this system is that viral infection can be easily monitored (Figure 1.6) by change in shape of the cells (cell volume increases and they become spherical) and nucleus becomes dotted (accumulation of viral particles). Since insect TOR and PDPK1 have been characterized and have been found functionally similar to their mammalian counterparts and S6K1 is found to be conserved with respect to similarity in structure and function primarily involved in regulating processes like cell size and aging in the system with rapamycin sensitivity identical to the kinase expressed in mammalian expression system (Watson et al., 1996, Stewart et al., 1996, Montagne et al., 1999, Alessi et al., 1997a). Therefore baculoviral expression of S6K1 in insect cell system reportedly produced a protein structurally and functionally homologous to its mammalian counterpart and also active towards phosphorylating 40S ribosomal protein S6 (Kozma et al., 1993).

1.10 S6K1 activation cascade and rationale of the study

S6Ks lie downstream of growth signalling pathways like mTOR & PI3 Kinase and are known to get activated in response to growth factor stimulation by a series of ordered phosphorylation events leading to kinase activation (Dennis et al., 1998, Mukhopadhyay et al., 1992, Mahalingam and Templeton, 1996). Its activation is reported to begin by a calcium dependent priming event to release the interaction between amino terminal and carboxy terminal domains (Hannan et al., 2003), which facilitates phosphorylation of several proline –directed serine residues in the pseudo-substrate domain possibly by MEK/Akt/CDK1 (Conus et al., 1998, Wang et al., 2001a). These phosphorylations open up the conformation to make Threonine 412 (T412) site in the hydrophobic motif accessible to mTOR kinase which is thought to mediate this phosphorylation (Isotani et al., 1999). This site is known to get

phosphorylated in a rapamycin sensitive manner possibly by mTOR (Pearson et al., 1995, Chung et al., 1992). In spite of the predicted model of rapamycin action, how rapamycin actually targets and carries over inhibition to S6K is not clearly understood (Saitoh et al., 2002), despite the discovery of TOR signalling motifs in S6K and its association with Raptor (regulatory associated protein of mTOR). It has been reported that these amino (FDIDL) and carboxy-terminal (RSPRR) signalling motifs mediate interaction with mTOR through raptor to phosphorylate S6K at Threonine 412 and carry rapamycin mediated inhibition to S6K (Schalm and Blenis, 2002, Schalm et al., 2005). Several independent studies suggest involvement of several other kinases known to phosphorylate this site like NEK, hVsp34, and DRAK2 etc (Belham et al., 2001, Byfield et al., 2005, Mao et al., 2009). A recent study has suggested this site to get phosphorylated by GSK3 and mTOR functions to maintain this phosphorylation by inhibiting PP2A (Shin et al., 2011). The final phosphorylation in the activation cascade is that of activation loop site (Threonine 252) which is done by PDPK1 (Phosphoinositide dependent protein kinase 1) resulting in full activation of the kinase (Pullen et al., 1998, Weng et al., 1995a, Alessi et al., 1998). The order of phosphorylation events still remains controversial as a recent study by Keshwani et al shows that T412 phosphorylation is not required for activation loop phosphorylation, and T252 phosphorylation precedes T412 which goes against the old view of the two being sequential and T252 follows T412 phosphorylations (Weng et al., 1998). Furthermore, the immunosuppressive agent rapamycin and its cellular receptor FKBP12, inhibit p70S6 kinase by causing selective loss of T412 phosphorylation, largely attributed to direct inhibition of TOR kinase although, recruitment/stimulation of phosphatase 2A also stands implicated in the process (Peterson et al., 1999). Turnover of phosphorylation at this site has therefore, been used as an index to monitor the activation state of the enzyme. Accordingly mutations replacing the individual residues to alanines, led to complete inactivation of the enzyme. While phospho-mimicking mutation at T412 reportedly produced enzyme with higher basal activity, no recovery was observed in T252E variant, due perhaps to the steric sensitivity of the region. Serine 394 (S394), a highly conserved residue was subsequently identified as another mitogen regulated phosphorylation site, whose mutation to alanine or aspartate brought about complete inactivation of the enzyme.

The three phosphorylations were later attributed to represent equivalent of conserved phosphorylations in the AGC subfamily of kinases, such that T252 represented the activation or T loop (AL), S394 the turn motif and T412 as the hydrophobic motif (HM) phosphorylation. With rapamycin insensitive proline directed MAP kinases known to phosphorylate the AID sites and the prospect of a similar kinase phosphorylating the turn motif site, the onus of S6K1 regulation has more or less been exclusively associated with AL and HM phosphorylations. While T252 at the AL was shown to be phosphorylated by a rapamycin insensitive phosphatidyl inositol dependent kinase 1 (PDK1) both in vivo and in vitro, mTOR kinase stands implicated in phosphorylating T412 at the HM. Both these phosphorylations exhibit a dramatic turnover upon activation and inhibition of the enzyme by rapamycin. Although rapamycin was shown to cause selective dephosphorylation at the HM site, the loss of AL phosphorylation associated with inhibition of the enzyme by rapamycin, has been attributed to its dependence on HM phosphorylation in accordance with the prevalent dogma. Interestingly, another model first proposed by Avruch and his co workers and recently refined by Taylor and colleagues, advocating occurrence of AL phosphorylation prior to HM phosphorylation, fails to account for the loss of AL phosphorylation in response to rapamycin.

In this study, we have attempted to understand the contribution of each phosphorylation in mediating rapamycin sensitivity of S6K1 individually and also as to how the turnover at each site relates to the turnover at the other. In addition, we also tried to explore the role of amino and carboxy terminal regulatory domains in mediating phosphorylation turnover at the two sites, to impart rapamycin sensitivity or their role in enzyme activation. We further analyzed the role of other pathways that reportedly regulate phosphorylation at these two sites to activate or inactivate the enzyme independent of TOR. In this approach, we used two local anaesthetics bupivacaine and lidocaine which reportedly inactivate ERK and Akt pathways; in the study our aim was to study the cross talk that may exist between S6K and ERK/Akt pathways and whether S6K is regulated by any input from them.

Cloning and Mutagenesis

S6K1 (p85), S6K1 (Δ2-46), S6K1 (ΔCT104), S6K1 (Δ2-46/ΔCT104), cloned in pMT2 vector at Ecor I site were a kind gift from Joseph Avruch at Harvard medical School (Boston MA). They were confirmed by digestion with Ecor I enzyme to get an appropriately sized band and further confirmed by sequencing of the plasmids.

2.1 Cloning of S6K1 (p85), S6K1 (Δ2-46), S6K1 (ΔCT104), S6K1 (Δ2-46/ΔCT104) in pVL-1392 for generation of recombinant viruses and subsequent expression in insect cell system

Amino-terminal Hemagglutinin tagged (HA) ribosomal protein S6 Kinase 1 (S6K1) and its amino and carboxy terminal truncated versions cloned in pMT2 vector at Ecor I site were a kind gif from Dr. Joseph Avruch (Harvard medical school, Boston, MA). pMT2 vector with S6K and its truncation variants were digested with Ecor I (NEB) at 37°C for 1 hr. Digested mixture was run on a 1.2 % Agarose gel ,bands (with insert) were gel cut and purified using gel extraction kit from Sigma . Ligation with Ecor I digested and CIP treated (calf intestine alkaline phosphatase) pVL-1392 vector was carried out at room temperature for 1hr using T4 DNA ligase from NEB (New England Bio labs) and various variants of the kinase i.e S6K1 (p85), pVL-1392 S6K1 (Δ2-46), pVL-1392 S6K1 (ΔCT104), pVL-1392 S6K1 (Δ2-46/ΔCT104).

Ligated mixture was transformed into DH5α using Transform Aid kit (Fermentas) as per manufacturer's protocol and grown on Ampicillin plates. Plasmids were isolated using plasmid mini-prep kit (Stratagene, USA) and digested with Ecor I to confirm the presence of insert and then further digested with Not I to check orientation of the Insert. For confirmation, clones formed were sequenced using services of Scigenom labs.

2.2 Generation of Mutants forms of S6 Kinase

Site directed mutagenesis was carried out to generate various point and truncations mutants using Quick change II mutagenesis kit (Stratagene) by a modified protocol described in Zhang *et al*. Reaction mixture was transformed into XL1-Blue chemically competent cells supplied with the kit by heat shock method on Ampicillin-LB Agar plate. Five transformants were selected at random for plasmid isolation and sent for sequencing. Mutagenesis efficiency was recorded at above 90%.

1. S6K (252 Threonine-Glutamic acid) in pMT2.

S6K variant carrying a point mutation at amino acid 252 in the catalytic domain, where threonine is changed to Glutamic acid to mimic phosphorylated residue.

Template used: pMT2-S6K

Primers used:

1. 5---GTCACGCACGAATTTTGTGGAACAATAGAATACATGGCCCCT---
2. 5---CCACAAAATTCGTGCGTGACTGTTCCATCATGAATAGATTC---3

Sequencing primers:

1. 5—CATGGAAGATACAGCTTGCTT---3
2. 5---CTGGGAAGAGCTTTTGGCTC—3

2. S6K (252 Threonine-Alanine) in pMT2

S6K variant carrying a point mutation at amino acid 252 ,where threonine is changed to Alanine to mimic de-phosphorylated amino acid residue.

Template used: pMT2-S6K

Primers used:

1. 5---GTCACGCACGCATTTTGTGGAACAATAGAATACATGGCCCCT---
2. 5---CCACAAAATGCGTGCGTGACTGTTCCATCATGAATAGATTC---3

3. S6K (412 Threonine-Glutamic acid) in pMT2.

S6K variant carrying a point mutation at amino acid 412 in the linker domain, where threonine is changed to Glutamic acid

Template used: pMT2-S6K

Primers used:

5---CTGGGTTTTGAATATGTGGCTCCATCTGTACTTGAAAGTG---3

5---GAGCCACATATTCAAAACCCAGAAAGACCTGGTTGGCACTTT—3

4. S6K (412 Threonine-Alanine) in pMT2

S6K variant carrying a point mutation at amino acid 412 in the linker domain, where threonine is changed to Alanine

Template used: pMT2-S6K

Primers used:

5---CTGGGTTTTGCATATGTGGCTCCATCTGTACTTGAAAGTGTG—3

5--- AGCCACATATGCAAAACCCAGAAAGACCTGGTTGGCACTTTC
ACTG---3

5. S6K (28 Phenylalanine-Alanine) in pMT2.

S6K variant carrying a point mutation at amino acid 28 in the amino-terminal domain where phenylalanine is changed to Alanine in the TOR signalling motif (TOS motif)

Template used: pMT2-S6K

Primers used:

1. 5---GAAGCTGAGGACATGGCAGGAGTGGCTGACATAGAC---3
2. 5 ---CTCTGGCTGGTCCAGGTCTATGTCCAGCCACTCCTGCC----3

6. S6K-ΔCT(28 Phenylalanine-Alanine) in pMT2

Carboxy-terminal truncated S6K variant carrying a point mutation at amino acid 28 in the amino-terminal domain, where phenylalanine is changed to Alanine in the TOR signalling motif (TOS motif).

Template used: pMT2-S6K-ΔCT

Primers used:

1. 5---GAAGCTGAGGACATGGCAGGAGTGGCTGACATAGAC---3
2. 5 ---CTCTGGCTGGTCCAGGTCTATGTCCAGCCACTCCTGCC----3

7. S6K -RR $_{(\Delta LEEGGQLN)}$ in pMT2.

S6K variant carrying an 8 amino acid deletion in the amino-terminal domain

Template used: pMT2-S6K

Primers used:

1. 5---GAAAGCATGGACCATGGGGG---3
2. 5---CTCATCCTCAGAGCCTGCATCC---3

8. S6K $_{(394\ Threonine\text{-}Glutamic\ acid)}$ in pMT2.

S6K variant carrying a point mutation at amino acid 394 where threonine is changed to Glutamic acid

Template used: pMT2-S6K

Primers used:

1. CTCGTCAGACACCTGTTGACGAACCCGATGAC---3
2. GAGTTGAGTCATCGGGTTCGTCAACAGGTG---3

9. S6K F28A -RR in pMT2.

S6K variant carrying both F28A and RR mutations

Template used: pMT2-S6K-RR

Primers used:

1. 5---GAAGCTGAGGACATGGCAGGAGTGGCTGACATAGAC---3
2. 5 ---CTCTGGCTGGTCCAGGTCTATGTCCAGCCACTCCTGCC----3

10. S6K (100-104 Lysine to Arginine) in pMT2.

S6K variant carrying Lysine to Arginine mutants from 100-104 residues

Template used: pMT2-S6K

Primers used:

1. 5---GCCATGGAGGGTGCTTAGAAGGGCAATGATAGTAAG ----3
2. 5---CATTGCCCTTCTAAGGACCCTCATGGCAAATATCTTCC ----3

11. S6K (299-300 Lysine to Arginine) in pMT2.

S6K variant carrying Lysine to Arginine mutants from 299-300 residues

Template used: pMT2-S6K

Primers used:

1. 5---GGAGAATAGAAGGGAGGACAATTGGACAAAATCCTC ----3
2. 5---GTCAAATTGTCCTTCCTTCTATTCTCCCCAGTGAATGGAG ----3

12. S6K (304-309 Lysine to Arginine) in pMT2.

S6K variant carrying Lysine to Arginine mutants from 304-309 residues

Template used: pMT2-S6K

Primers used:

1. 5---CAATTGACAGAATCCTCAGATGTAGACTTAATTTGCCT ----3
2. 5---CAAATTAAGTCTACATCTGAGGATTCTGTCAAATTGTCTTT ----3

13. S6K (325-329 Lysine to Arginine) in pMT2

S6K variant carrying Lysine to Arginine mutants from 325-329 residues

Template used: pMT2-S6K

Primers used:

1. 5---GATCTGCTTAGAAGGCTGCTGAGGAGAAATGCTGCTTC ----3
2. 5---GCATTTCTCCTCAGCAGCCTTCTAAGCAGATCTCGAGC----3

14. S6K 412E/252E

S6K variant carrying a double mutation one at position 412 (Thr to Glu) and another at amino acid 252, where threonine is replaced by Glutamic acid

Template used: pMT2-S6K-412E

Primers used:

1. 5--GTCACGCACGAATTTTGTGGAACAATAGAATACATGGCCCCT---3
2. 5---CCACAAAATTCGTGCGTGACTGTTCCATCATGAATAGATTC---3

15. S6K412A/252A

S6K variant carrying a double mutation one at position 412 (Thr to Ala) and another at amino acid 252, where threonine is also replaced by Alanine

Template used: pMT2-S6K-412A

Primers used:

1. 5--GTCACGCACGCATTTTGTGGAACAATAGAATACATGGCCCCT---3
2. 5---CCACAAAATGCGTGCGTGACTGTTCCATCATGAATAGATTC---3

16. S6K412A/252E

S6K variant carrying a double mutation one at position 412 (Thr to Ala) and another at amino acid 252, where threonine is also replaced by Alanine

Template used: pMT2-S6K-412A

Primers used:

1. 5---GTCACGCACGAATTTTGTGGAACAATAGAATACATGGCCCCT---3
2. 5---CCACAAAATTCGTGCGTGACTGTTCCATCATGAATAGATTC---3

17. S6KF28A/252A

S6K variant carrying a double mutation one at position 28 (Phe to Ala) and another amino acid 252, where threonine is also replaced by Alanine

Template used: pMT2-S6K-F28A

Primers used:

1. 5--GTCACGCACGCATTTTGTGGAACAATAGAATACATGGCCCCT---3
2. 5---CCACAAAATGCGTGCGTGACTGTTCCATCATGAATAGATTC---3

3. S6K412E/394E

S6K variant carrying a double point mutation one at amino acid 412(Thr - Glu) in the linker domain and another at position 394 where Serine is replaced with Glutamic acid

Template used: pMT2-S6K-394E

Primers used:

1. 5---CTGGGTTTTGAATATGTGGCTCCATCTGTACTTGAAAGTG---3
2. 5---GAGCCACATATTCAAAACCCAGAAAGACCTGGTTGGCACTTT—3

2.3 Cloning of S6K point mutants in pVL-1393 for generation of recombinant viruses and subsequent expression in insect cell system

S6K point mutants were cloned in pVL-1393 for virus generation and expression analysis as described for cloning truncation mutants. Site directed mutagenesis was carried out in mammalian expression vector (pMT2) and confirmed by sequencing. After sequence confirmation, digested with Ecor I and ligated with CIP treated pVL-1393 digested with same enzyme (Ecor I) using T4 DNA ligase and transformed into XL1-blue chemically competent cells and ligation confirmed with digestion Ecor I and orientation of the insert confirmed by digestion with Not I enzyme.

2.4 Cloning and bacterial expression of S6 (C-Terminal 69 amino acids) in pGEX-4T2 as a GST fusion protein

Carboxy terminal 210 Bp sequence corresponding to 69 amino acids of ribosomal protein S6 was amplified from its cDNA cloned in pDNR vector (pDNR-S6) by standard PCR method. Primers were designed to carry 5′-Ecor I and 3′-Xho I in the amplified fragment and were in frame with the GST (Glutathione S transferase) coding region of the vector.PCR reaction was run for 35 cycles at annealing temperature of 56°C.

Forward primer:

5--CGCCGAATTCTGACTCCACGTGTCCTGCAGCACAAAC----3

GAATTC-EcorI Site

Reverse primer:

5-----CGCGCCTGCTCGAGTTATTTCTGACTGGATTCAGAG----3

CTCGAG-XhoI site

PCR amplified DNA was mixed 6X loading dye (Fermentas) and the sample was electrophoresed on 2% Agarose gel, and its size was determined by comparison to the mobility of 100 Bp DNA ladder. Respective bands were gel cut and DNA was purified by gel purification kit (Sigma) according to manufacturer's protocol.

2.4.1 Restriction digestion of the S6 DNA

Purified product was digested with Ecor I and Xho I overnight at 37°C and digested product was purified using spin column based purification kit (Fermentas) according to manufacturers protocol.

2.4.2 Restriction digestion of the pGEX-4T2

pGEX-4T2 vector was digested with the same enzymes under same conditions and digested vector purified using gel purification kit form Sigma

2.4.3 Ligation Reaction

Vector DNA and insert (digested S6 DNA) were mixed in a ratio of 1:10 by mass (so as to match number of molecules) in presence of T4 DNA ligase. The contents were vortexed and spun down for 3-5 seconds and incubated at room temperature for 30 minutes.

2.4.5 Transformation

5µL of ligation mixture was transformed into 50 µL of BL21-DE3 cells by Transform Aid Kit (Fermentas) according to manufacturer's instructions and plated on Ampicillin containing LB agar and incubated overnight at 37ºC. Ampicillin resistant clones were picked up and digested with same restriction enzymes as used for cloning the insert to confirm the presence of insert. Five colonies were taken at random from the plate and induced using IPTG, run on a 12% SDS PAGE gel to check induction of the recombinant GST fusion protein. It was purified using GSH Agarose beads and eluted using reduced glutathione buffer for subsequent use in kinase assays.

2.5 Cloning and baculoviral expression of constitutively active PDPK1 (3-Phosphoinositide-dependent protein kinase 1)

Catalytic domain sequence corresponding to 531 Bp of PDPK1 coding for catalytic domain 177 amino acids was amplified by PCR using pDNR-PDPK1 as template. Forward primer carried BamH I site and Ecor I site in the reverse primer for cloning and expression in pKmyc vector in frame with amino terminal myc tag. PCR reaction was run for 30 cycles at an annealing temperature of 58°C.

Forward primer:

5—CGCGGGATCCATGGACGGCACTGCAGCCGAG----3

GGATCC-BamH I Site

Reverse primer:

5-----CGCCGAATTCAGCGGTGAGCTTCGGAGGCGT----3

GAATTC-Ecor I Site

2.5.1 Restriction digestion and cloning of PDPK1 (ΔNH 26/ΔCT197) - cDNA

PCR amplified DNA was mixed with 6x loading dye and the sample was electrophoresed on 2% agarose gel, and its size (551Bp) was determined by comparison to the mobility of 100 Bp DNA ladder. Respective bands were gel cut and DNA was purified by gel purification kit (Sigma) according to manufacturer's protocol.

2.5.2 Restriction digestion of the PDPK1 DNA and pKmyc Vector

Digestion of the amplified fragment and pKmyc vector was done with BamH I and Ecor I at 37ºc overnight. Digested product was run on a 1.2% agarose gel, bands were gel cut and purified by DNA purification kit (Sigma) according to manufacturer's protocols

2.5.3 Ligation Reaction

Vector DNA and insert (digested ΔPDPK1 DNA) were mixed in a ratio of 1:10 by mass (so as to match number of molecules) in presence of T4 DNA ligase. The contents were vortexed and spun down for 3-5 seconds and incubated at room temperature for 30 minutes.

2.5.4 Transformation

5μL of ligation mixture was transformed into 50 μL of DH5α cells by Transform Aid Kit (Fermentas) according to manufacturer's instructions and plated on Ampicillin containing LB agar and incubated overnight at 37ºC. Ampicillin resistant clones were picked up and plasmids were purified using plasmid purification kit (Stratagene) and digested with same restriction enzymes as used for cloning the insert to confirm the presence of insert. This was further confirmed by sequence analysis of the vector along with the insert.

2.6 Sub-cloning of ΔPH-PDPK1 in pVL-1393

Amino-terminal myc-tagged ΔPH-PDPK1 cloned in pKmyc was used as a template to amplify it along with the tag for cloning in pVL-1393 and baculoviral expression. Following primers were used with highlighted restriction sites.

5---CGATGCGCCGCATGGAACAGAAACTCATCTCTGAAG---3

GCGCCGC-Not I Site

Reverse primer:

5-----CGCCGAATTCAGCGGTGAGCTTCGGAGGCGT----3

GAATTC-Ecor I Site

For PCR amplification, above shown primers were used to amplify the clone at annealing temperature of 56°C. PCR reaction mixture was run on a 1.2% to confirm amplification of the cDNA.

2.6.1 Restriction digestion of the ΔPDPK1 cDNA and pVL-1393 Vector

Digestion of the amplified fragment and the vector was done with Not I and Ecor I at 37ºC for 16 hrs (overnight). Digested products were purified by DNA purification kit (Fermentas) according to manufacturer's protocol. Ligation was done at same conditions as shown above.

2.6.2 Transformation

Ligated mixture was transformed into chemically competent DH5α cells and confirmation of cloning done by sequencing.

2.6.3 Baculoviral Expression

Recombinant pVL-1392 with PDPK1 was transfected into Sf9 cells along with the linearized baculoviral DNA for recombinant protein expression using Baculogold kit (BD Biosciences)

2.7 Cloning and Baculoviral expression of constitutively active mTOR

mTOR (mechanistic target of rapamycin) cloned in pKmyc was obtained from Addgene Inc. USA. For its constitutive activation, point mutations in residues S2215Y were introduced by a PCR based site directed mutagenesis protocol (Quick change) as per manufacturer's instructions using primers as shown below:

5---AACACCCTTCTGAATGACCCAACATATCTTCGGAAA---3

5---GACGCTGAGGTTTTTCCGAAGATATGGGTCATT---3

PCR mixture was directly transformed into chemically competent XL1-Blue cells on Ampicillin LB-agar plates and five colonies were picked at random for plasmid isolation (Stratagene) according to manufacturer's instructions and success of mutagenesis was confirmed by sequencing (Scigenom labs).

Mutagenised mTOR was digested with Ecor I and Not I for 1 hr at 37°C and mTOR fragment was then ligated with pVL-1392 (digested with same two enzymes) and transformed into chemically competent XL1-Blue cells. Ampicillin resistant clones were picked to check cloning of insert. pVL-mTOR was then transfected with linearized baculoviral DNA into Sf9 cells for virus generation.

2.8 Generation of recombinant viruses: Recombinant viruses expressing various proteins in insect cell system were generated using Baculo gold kit (BD Biosciences) as per the manufacturer's protocol. Briefly cDNA encoding various proteins cloned in either pVL1392 or pVL-1393 were co-transfected with linearized baculoviral DNA into insect cells using transfection reagent provided with the kit. cDNA cloned in the transfer vector gets recombined inside the cells with the linearized baculoviral DNA. Expression of the recombinant protein is under the control of strong polyhedrin promoter, expression this gene is not required when cells are grown in culture. This promoter is maximally expressed very late in infection and can be used for expression of very toxic proteins. Many post-translational modifications are made correctly producing proteins in native form.

2.9 Protein expression and purification: Constructs pGEX4T2 (GST-S6) and the parental control plasmid pGEX4T2 were transformed into the BL21-DE3 pLysS host strain (Sigma) and fusion protein expression was induced for 16hrs at 30 °C with 1mM IPTG. Bacteria were collected at 5,000g and cell lysates prepared using a protocol described in Harper et al (6.6.1-Current protocols in protein science). Briefly, cells were lysed in buffer containing Tris-Cl (50mM), EDTA(5mM), NaCl(50mM), lysozyme (0.2mg/mL) and protease inhibitor cocktail (Sigma) at pH 8.0 and incubated on ice for 30 minutes followed by sonication (10 Sec pulses -10 Sec rest×10). Pre cleared lysates were subjected to affinity chromatography using GSH beads (50% slurry) by batch purification method as per standard conditions as recommended by the manufacturer (Sigma Aldrich). Fusion protein was eluted from the beads using glutathione buffer containing Tris-Cl (50mM), 10mM reduced glutathione (Sigma) and SDS-PAGE was used to assess the purity of the protein. Purified protein was desalted and glutathione removed by dialysis against kinase buffer at 4°C overnight.

2.10 Cell culture: HEK-293 cells were cultured in Dulbecco's modified Eagles medium (with appropriate antibiotics) containing 10% (v/v) fetal bovine serum with 50 μg/ml penicillin and 100 μg/ml streptomycin (Sigma Aldrich) in a humidified atmosphere of 5% CO2/95% air at 37°C. Cells were routinely passaged twice a week. Sf9 cells were obtained from Sigma and Invitrogen and grown routinely in TNM-FH insect media and Grace's media (Invitrogen) supplemented with 5% fetal bovine serum , 50 μg/ml penicillin and 100 μg/ml streptomycin (Sigma Aldrich) in a non-humidified incubator at 28°C.

2.11 Transfections: Transfections were carried out by standard calcium phosphate method. Briefly, cells at a confluency of 70 % were incubated with DNA-phosphate complex ($CaCl_2.6H_20$+DNA+HBS) overnight. Cells were then grown for 48 hrs and expression monitored by western blotting. Alternatively, transfections were carried out using Lipofectamine reagent (Invitrogen Carlsbad, California) as per manufacturer's instructions.

Insect cells (Sf9) were cultured in serum free TNM-FH media or Grace's media and seeded at cell density of $2x10^6$ cells per 60mm tissue culture plate and transfected

with individual viruses for each of the above mentioned constructs with multiplicity of Infection (M.O.I.) of ≤1 for 58-60 hours.

2.12 Immuno-precipitations: HEK-293 cells were starved overnight in DMEM containing 2% serum. HEK-293 and Sf9 cells were exposed to rapamycin (50ng/ml) for 20 minutes before harvest. Cells were lysed on ice in a buffer containing 50mM Tris-Cl (pH 7.5), 10mM MgCl2, 5mM EDTA, 2mM DTT, 50mM β-Glycero-phosphate, 0.5% Triton X-100 and Protease inhibitor cocktail (Sigma) for 30 minutes and centrifuged at 15000g for 30 minutes to clear the lysate. Cleared lysate was incubated with Anti-HA antibody immobilized on protein G- Agarose (Calbiochem) beads overnight. Beads were washed thrice with lysis buffer containing 500mM NaCl and a final wash with kinase buffer to remove the salt.

2.13 Immuno-complex kinase assays and Western blotting: S6Kα1WT and its various truncation mutant versions immobilized on HA-beads were incubated with 1μg GST-S6 and 5μCi P^{32}ATP in a kinase reaction buffer containing 50mM Tris-Cl (pH 7.0), 10mM $MgCl_2$, 0.5mM DTT, 50mM β-Glycero-phosphate, and 1mM ATP for 20 minutes at 37°C. Reaction was stopped by adding 6X loading buffer, run on a 12% SDS-PAGE gel. Proteins were transferred on PVDF membrane, auto-radiographed, probed with different antibodies at indicated concentrations and analyzed using Odyssey infrared imager (LI-COR).Alternatively,S6 peptide was used as a substrate and activity determined by standard protocol .

For western blotting proteins were run on 12% SDS-PAGE gel and transferred on PVDF membranes using wet blotting procedure at 70V constant current for 90 minutes. Membranes were washed three times with PBS for 10 minutes each and blocked for 60 min with ODYSSEY blocking buffer (LI-COR biotechnologies USA). Primary antibodies were diluted 1:1000 (anti-S6K; Santacurz), 1:1000 (anti-phospho T412; Cell signalling technologies), 1: 500 (anti-Phospho T252; R&D Bio systems, USA), 1:1000 (anti-beta tubulin; Sigma) in 0.5 % blocking solution and incubated overnight at 4°C on a rocking plate. Thereafter, membranes were washed three times with PBS-T (PBS with 0.05% Tween 20), followed by an incubation for 1 hr at RT with a 1:10,000 dilution of Infrared dye conjugated goat anti-rabbit secondary

antibody (800CW) or 1:10000 dilution of Infrared dye conjugated goat anti-mouse secondary antibody (680CW). After subsequent washes with PBS-T, antibody binding was visualized by imaging on LICOR ODYSSEY infrared system. For re probing, blots were stripped in stripping buffer (10 mM glycine, 2% SDS, pH 2.0) and incubated for 30 minutes at room temperature with occasional agitation. Membranes were washed for 2 x 10 minutes in PBS-T at room temperature using large volumes of washing buffer. Membranes were then blocked and processed the same way as above.

2.14 Phosphatase assays: Active S6Kα1 WT as HA-conjugate with protein G-beads were washed thrice with PIPES buffer and then incubated with varying concentrations of Potato acid Phosphatase in PIPES buffer, containing 20mM PIPES (pH 4.8), 20mM KCl, 1mM DTT,1mM $MgCl_2$ at 37°C for 30 minutes and then washed thrice with phosphatase inhibitor buffer containing 50mM Tris-Cl (pH 7.0), 10mM Sodium Fluoride , 1mM Sodium Ortho-Vanadate, 2mM Sodium Pyrophosphate deca-hydrate and 50mM β-Glycero-phosphate and once with kinase buffer. PP2A assay was done similarly in a buffer containing 20 mM MOPS, pH 7.5, 60 mM 2-ME, 0.1 M NaCl, and 0.1 mg/ml serum albumin) in a reaction volume of 50µl and reaction was stopped with 5ηM okadaic acid.

2.15 In-cell western assays and fluorescence quantitation: In cell western assays were done according to the protocol provided by LI-COR biotechnologies. Briefly, NIH-3T3 cells seeded in 12 well plates were transfected with pMT2-HA-S6K1 and treated with inhibitory concentrations of various drugs 48 hrs post transfection. For analysis, cells were washed with PBS, fixed using 4% p-formaldehyde, permeabilized using 0.1%Triton X-100 in PBS, blocked and then incubated with Anti-phospho-S6K antibodies in LI-COR blocking reagent overnight and average florescence intensity (in arbitrary units) of each well quantitated using Odyssey infrared imager (LI-COR Biotechnologies).

Results

Part I

Cloning , Mutagenesis and Expression

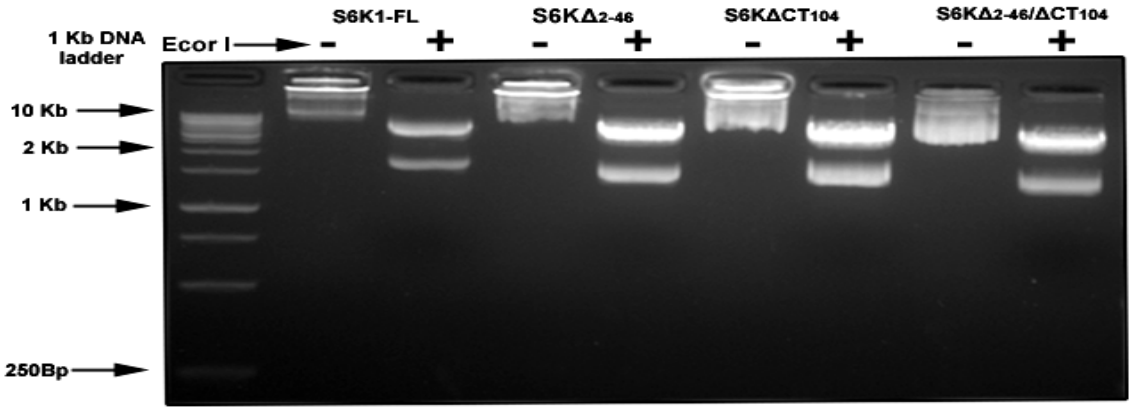
The ribosomal protein S6 kinases, S6K1 and S6K2 are two highly homologous serine/threonine kinases that are activated in response to growth factors, cytokines and nutrients. The S6 kinases have been linked to diverse cellular processes, including protein synthesis, mRNA processing, glucose homeostasis, cell growth and survival. Studies in model organisms have highlighted the roles that S6K activity plays in a number of pathologies, including obesity, diabetes, ageing and cancer. Our study was focused to understand regulation of p85S6K1 (S6K1) and analysis of domain specific inputs that together carry rapamycin inhibition and activation in response to growth factors and mitogens. Since several phosphorylation sites and domains specific for interaction with various cellular regulators have been characterised in mammalian cell system. We analysed those mutants for their potential to regulate S6K1 activity in comparison with baculoviral expression system. Insect cell line as a model system was chosen because activated state cannot be disengaged from basal state of the enzyme (S6K1) in mammalian cell system as even after considerable serum starvation; it is not possible to take cells to G_0. Another important character with baculoviral expression and insect cell system is that the foreign proteins undergo correct post translational modifications comparable to mammalian cell system producing biologically active proteins. So it was considered an ideal system to study dynamics of critical phosphorylation sites and domain specific inputs that together produce an active enzyme.

p85S6K1 and its domain truncation mutants cloned in pMT2 were as a kind gift from Dr Joseph Avruch (Harvard medical school, Boston). They were transferred into pVL-1392 for baculoviral expression and all other point mutants were generated using Quick change$^{TM\ site}$ directed mutagenesis kit in pMT2 vector and then transferred to pVL-1393 vector for subsequent baculoviral expression.

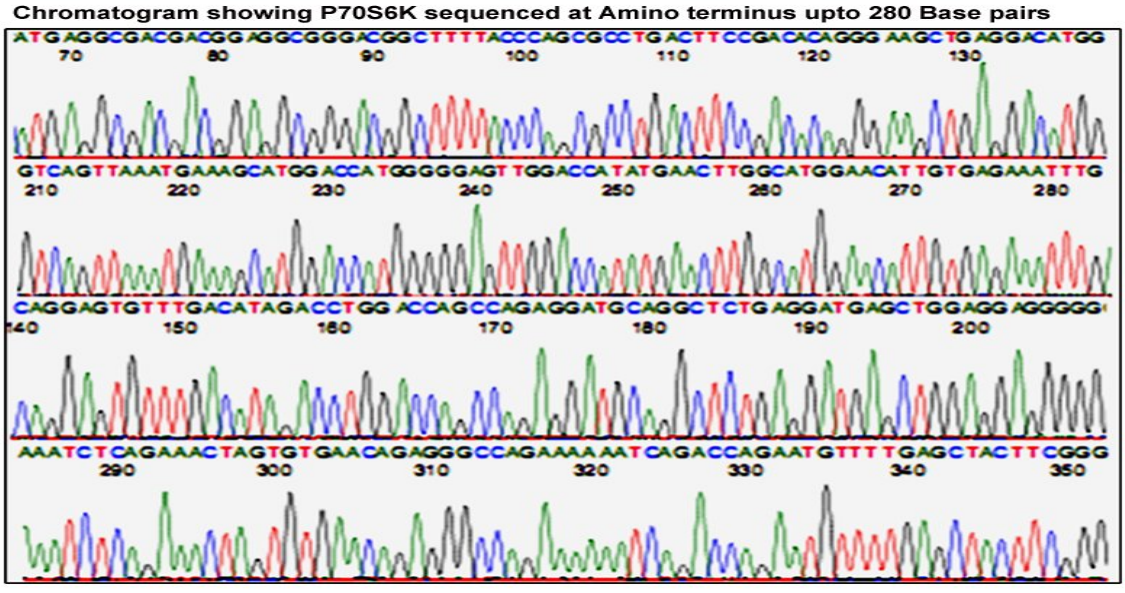
3.1 Restriction digestion of pMT2-S6K1 along with truncation mutants and sequence analysis for confirming authenticity of the clones.

p85-S6K and varous truncation mutants cloned in pMT2 vector at EcorI site were digested with the enzyme (EcorI) to confirm the clones and were run of an 1% Agarose gel. They were further checked for internal deletions if any and for overall authenticity by sequence analysis .

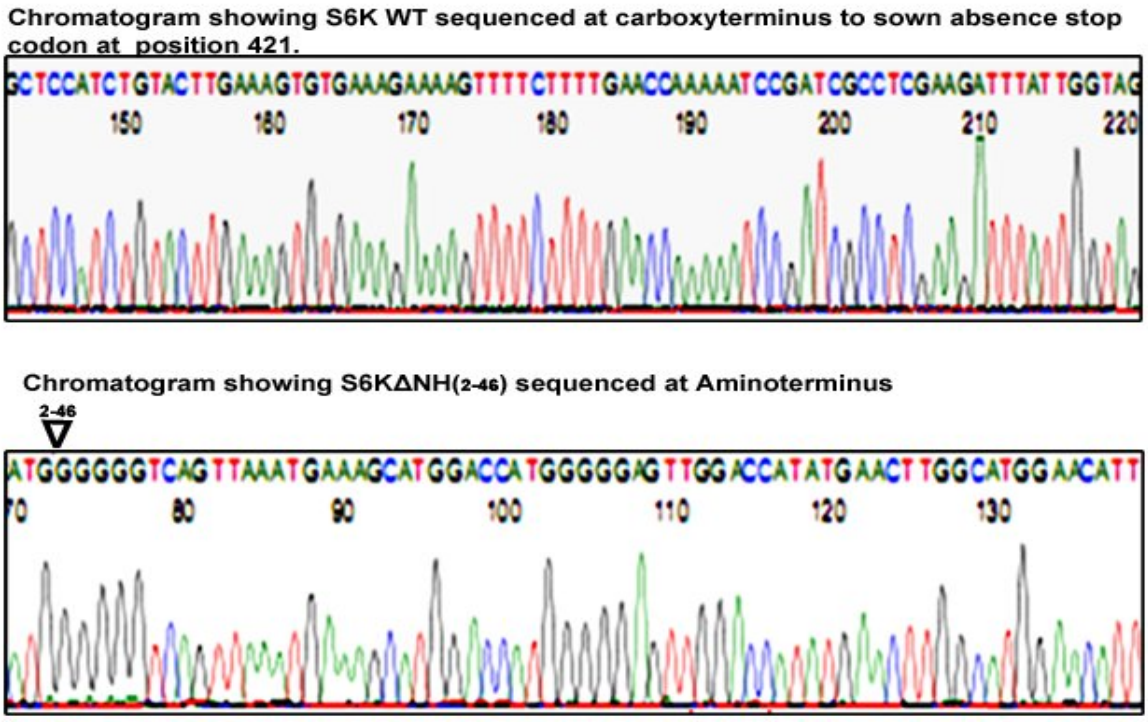
a)



b)



c)



d)

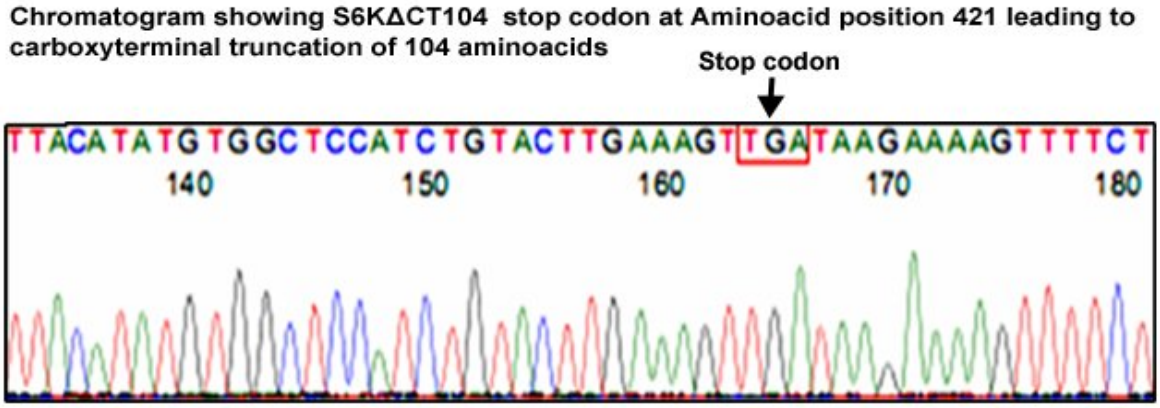
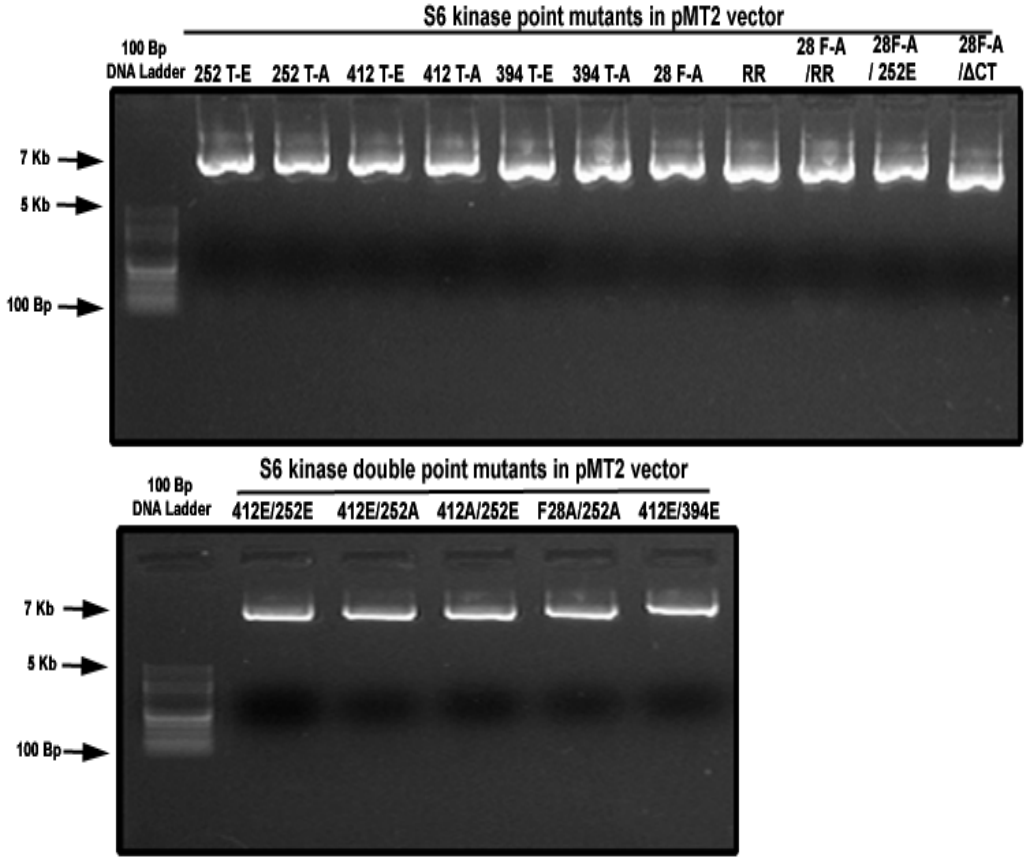


Figure 3.1 Restriction digestion of pMT2-S6K1 along with truncation mutants and sequence analysis for confirming authenticity of the clones. **(a)** S6 kinase and various truncation mutants cloned in pMT2 vector provided by Dr.Joseph Avruch, were digestd with EcorI and run on 1% Agarose gel **(b)** Chromatogram showing sequence analysis of S6K full length cDNA sequenced at its aminoterminus **(c)** Chromatogram showing sequence analysis of S6K full length cDNA sequenced at its carboxyterminus and $\Delta_{2\text{-}46}$S6K cDNA sequenced at its aminoterminus (panel2) **(d)** Chromatogram showing sequence analysis of S6KΔCT104 cDNA sequenced at its carboxyterminus showing stop codon at position 421.

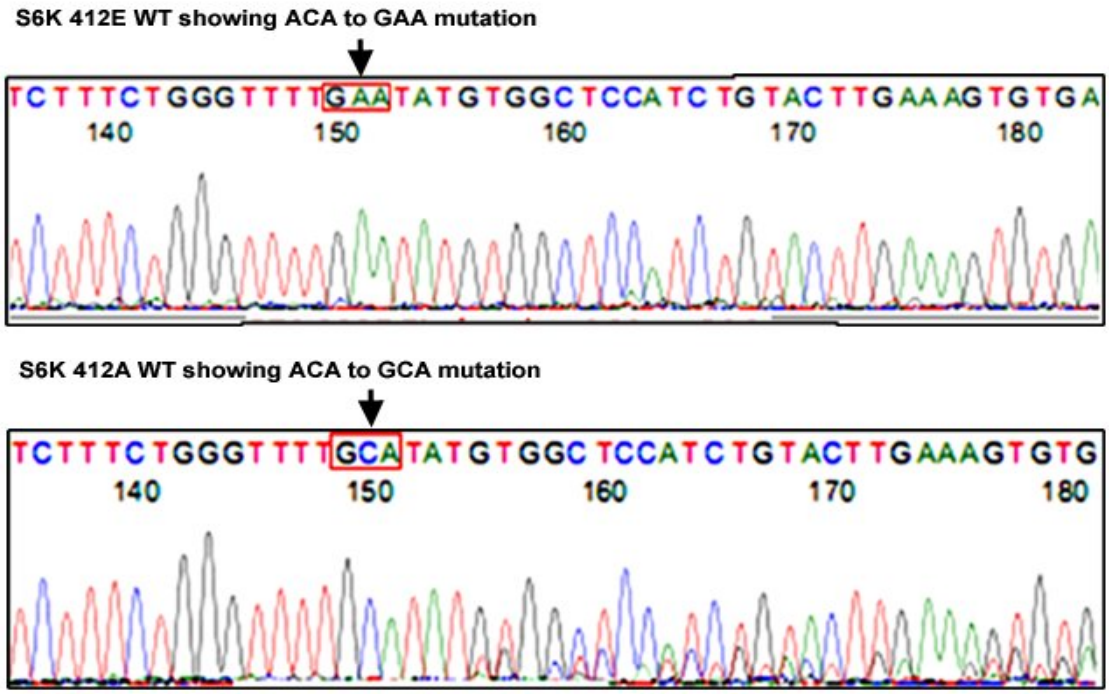
3.2 Site directed mutagenesis

Various point and deletion mutants were constructed by a PCR based method using Quick Change ™ (Stratagene Inc, USA) by a modified protocol as described in Zhang *et al*.10 µL of the PCR reaction mixture after DpnI digestion (which cleaves methylated parental plasmid DNA) was run on a 1% Agarose gel , to confirm amplification of the plasmid DNA with possible mutations. 5 µL of the mixture was transformed into chemically competent XL1-Blue cells supplied with kit by heat shock method , plated on ampicillin LB-agar . Ampicilin resistant colonies (5 in mumber) were picked at random and plasmid DNA isolated. Mutagenesis was confirmed by sequence analysis using services of Scigenom Labs Pvt. Ltd (Kerala,India). Mutagenesis effeciency was recorded at above 90%.

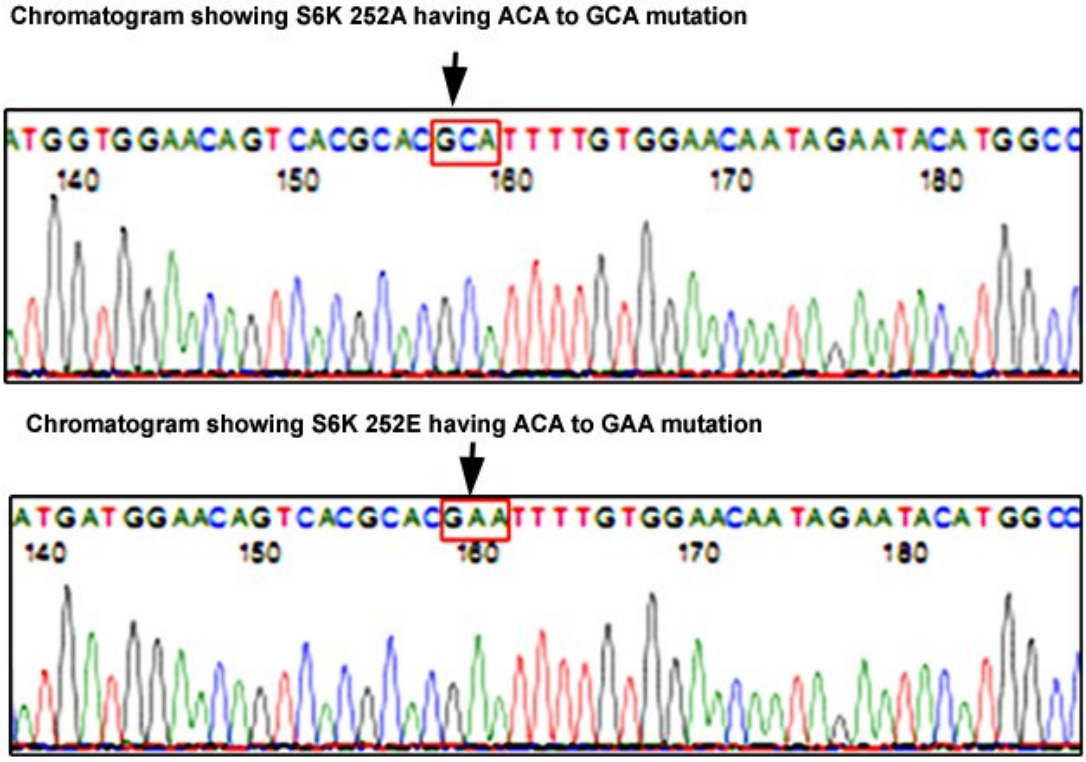
a)



b)


S6K 412E WT showing ACA to GAA mutation
TCTTTCTGGGTTTTGAATATGTGGCTCCATCTGTACTTGAAAGTGTGA
140
150
160
170
180
S6K 412A WT showing ACA to GCA mutation
TCTTTCTGGGTTTTGCATATGTGGCTCCATCTGTACTTGAAAGTGTG
140
150
160
170
180

c)


Chromatogram showing S6K 252A having ACA to GCA mutation
ATGGTGGAACAGTCACGCACGCATTTTGTGGAACAATAGAATACATGGCC
140
150
160
170
180
Chromatogram showing S6K 252E having ACA to GAA mutation
ATGATGGAACAGTCACGCACGAATTTTGTGGAACAATAGAATACATGGCC
140
150
160
170
180

d)

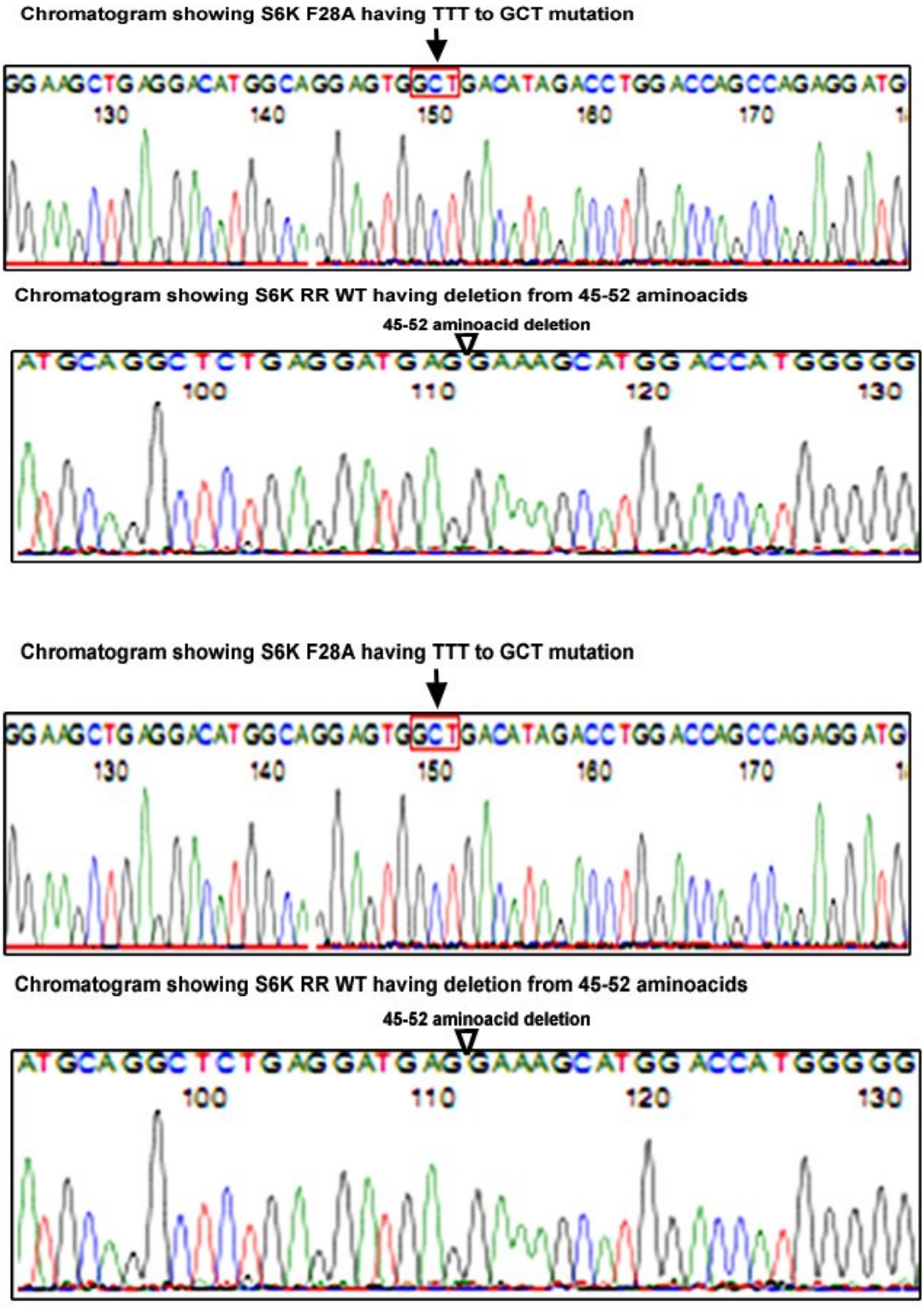

Chromatogram showing S6K F28A having TTT to GCT mutation
GGAAGCTGAGGACATGGCAGGAGTGGCTGACATAGACCTGGACCAGCCAGAGGATG
130
140
150
160
170
Chromatogram showing S6K RR WT having deletion from 45-52 aminoacids
45-52 aminoacid deletion
ATGCAGGCTCTGAGGATGAGGAAAGCATGGACCATGGGGG
100
110
120
130
Chromatogram showing S6K F28A having TTT to GCT mutation
GGAAGCTGAGGACATGGCAGGAGTGGCTGACATAGACCTGGACCAGCCAGAGGATG
130
140
150
160
170
Chromatogram showing S6K RR WT having deletion from 45-52 aminoacids
45-52 aminoacid deletion
ATGCAGGCTCTGAGGATGAGGAAAGCATGGACCATGGGGG
100
110
120
130

Figure 3.2: Agarose gel picture showing mutagenised S6 Kinase (cDNA) in pMT2 vector . Mutagenesis was carried out using Quick change ^{TM} kit and 5µL of the amplified product was run on a 1% Agarose gel and mutagenesis confirmed by sequence analysis. **(a)** Mutagenised S6 kinase with desired Mutations (as shown) run on a 1% Agarose gel and confirmed by sequencing **(b)** Chromatogram showing ACA to GAA mutation replacing Threonine with Glutamic acid at position 412 in the polypeptide and ACA to GcA mutation replacing Threonine with alanine at te ssame position (second panel) **(c)** Chromatogram showing ACA to GAA mutation replacing Threonine with Glutamic acid at position 252 in the polypeptide and ACA to GCA mutation replacing Threonine with alanine at the same position (second panel) **(d)** Chromatogram showing TTT to GCT mutation replacing Phenyalanine with Alanine at position 28 in the polypeptide and a deletion mutant (RR) carrying 21 Bp deletion (45-52 aminoacids) (lower panel).

3.3 Sub cloning of these mutants into pVL-1393 Vector for baculoviral expression

All these mutants generated in pMT2 vector were transferred into pVL-1393 vector. Briefly, p85S6K1 with desired mutations were digested with Ecor I along with HA-tag and ligated with EcorI digested and CIP treated pVL-1393 vector for subsequent baculoviral expression.

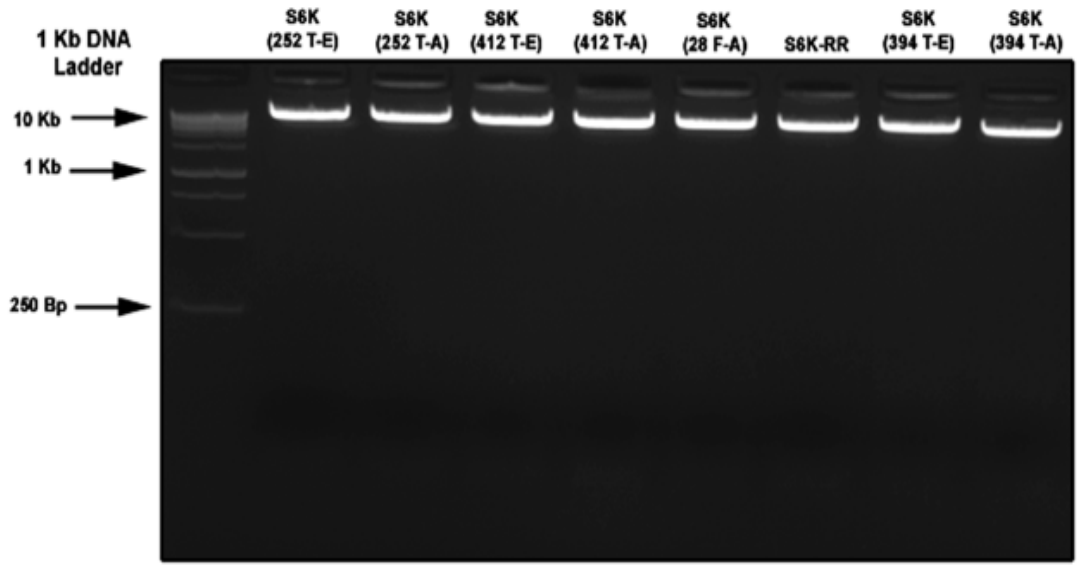


Figure 3.3: Agarose gel showing various point mutants cloned in pVL-1393 for virus generation.

3.4 Cloning of constitutively active PDPK1 in mammalian expression vector and baculoviral transfer vector for subsequent virus generation

S6K is known to get activated by several cellular factors in response to growth factors and mitogens. One of the kinases that phosphorylate it at the activation loop (AL) is PDPK1. Since insect version of this kinase (PDPK1) has been cloned and characterised, however S6K expressed in Sf9 cells by baculoviral infection lacked activation loop phosphorylation (T252) known to be mediated by PDPK1. Therefore in order to study, whether it is inactivated as a result of virus infection or some other mechanism leading to S6K not being phosphorylated at the activation loop. It was desired to clone and express constitutively active PDPK1 in Sf9 system for *in vitro* kinase assays using S6 kinase as a substrate. Constitutively active PDPK1 (ΔPH-PDPK1) was generated by PCR amplification of catalytic domain 531 Bp cDNA using full length PDPK-pDNR as template with primers having 5' BamH I and 3' EcorI in the amplified product. PCR amplified product was purified and digested with these two enzymes for compatible in frame cloning with amino terminal myc tag in pKmyc vector using T4 DNA ligase. Cloning of the insert was confirmed by restriction digestion and sequence analysis. pKmyc-ΔPH-PDPK1 was then used as a template to amplify this cDNA coding for constitutively active protein with primers having 5' Not I and 3' Ecor I in the amplified product for cloning in pVL-1393 for baculoviral expression and amplified product was digested and ligated with pVL-1393 vector digested with same two enzymes using T4 DNA ligase. Cloning was confirmed by restriction digestion of the recombinant plasmid. Myc tagged PDPK1 was used for virus generation as described in methods section by standard protocol and used for further experimentation.

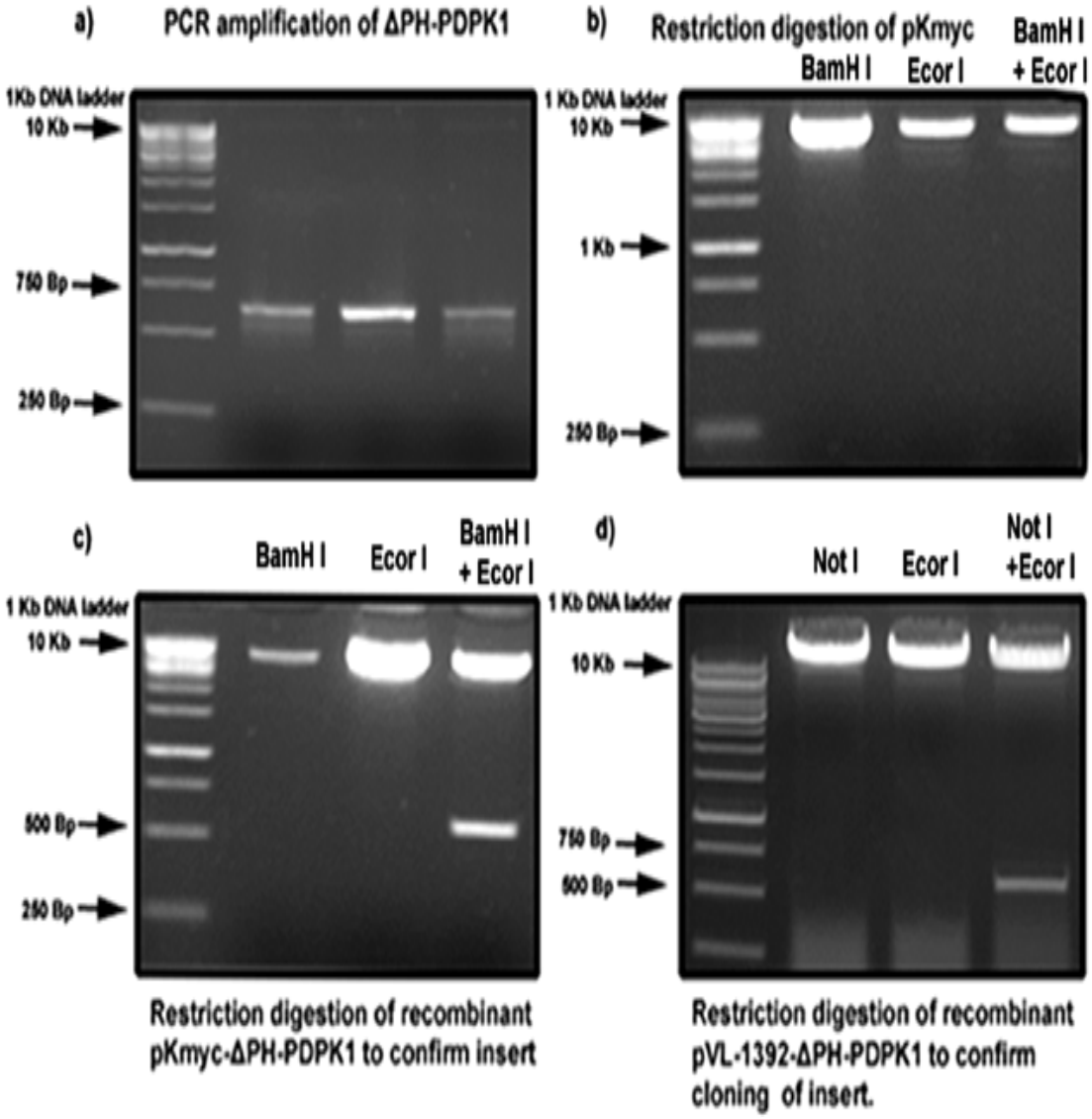
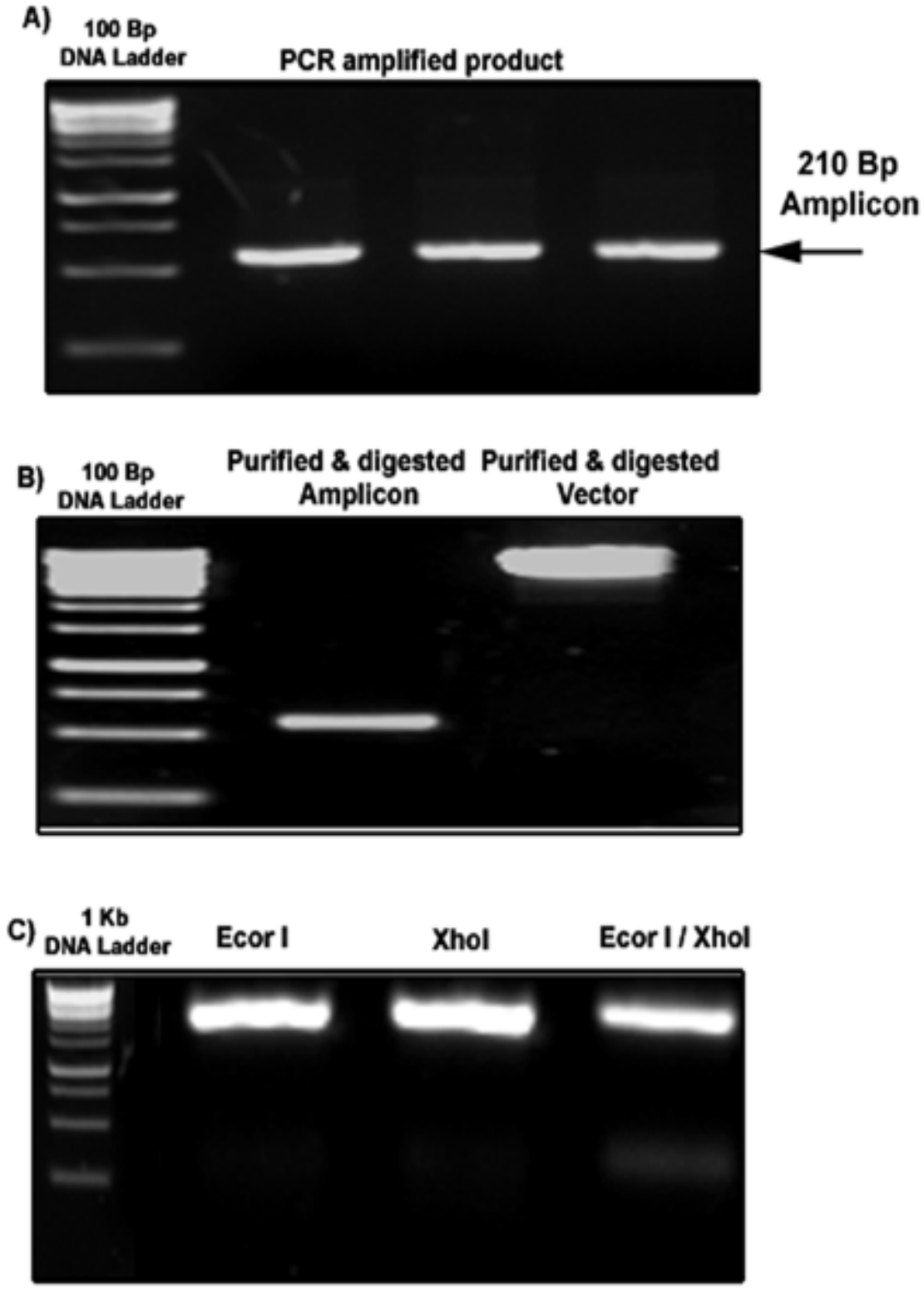


Figure 3.4: Cloning of ΔPH-PDPK1 in pKmyc and pVL-1393 (a) 1.5% Agarose gel showing PCR amplified 550 Bp fragment corresponding to ΔPH-PDPK1 **(b)** 1% Agarose gel showing restriction digestion of pKmyc vector with shown enzymes **(c)** 1% Agarose gel showing cloning of ΔPH-PDPK1 in pKmyc vector **(d)** 1% Agarose gel showing cloning of ΔPH-PDPK1 in pVL-1393 vector .

3.5 Cloning and expression of ribosomal protein S6 as a GST fusion protein in bacterial expression system

S6 is a 40S ribosomal subunit protein which is known to get phosphorylated at several sites in response to growth factors and mitogens. These phosphorylations are catalyzed by several kinases like S6 kinase family, RSKs and MAPK. However, the phosphorylation of critical and evolutionarily conserved carboxy terminal sites S235, S236, S240, S244, and S247 have been mapped to S6K1. Phosphorylation at these sites is a direct index of S6K1 activation, which in turn governs several cellular processes. Therefore, cloning, bacterial expression and purification of this protein was desired for use *in vitro* S6 kinase assays. We attempted to express full length version of this protein in bacterial expression system in the first instance but it was not possible to purify it due to its insoluble character. Then we PCR amplified cDNA (cloned in pDNR vector) corresponding to carboxy terminal 69 amino acids containing S6 kinase mediated phosphorylation sites using primers having 5' EcorI and 3' XhoI in the amplified product. Digestion of the purified product was carried out with these two enzymes and ligated with pGEX-4T2 (digested with same two enzymes) in frame with GST using T4 DNA ligase. 5µL (5/20) was transformed into chemically competent BL21-DE3 (pLysS) cells for bacterial expression and purification using GSH-Agarose (Sigma). Protein induction and purification was monitored on a 12% SDS-PAGE gel. Purified protein was eluted from the beads using glutathione containing elution buffer and purified protein was dialysed against kinase buffer at 4ºC overnight for salt removal for its subsequent use *in vitro* S6 kinase assays.


A)
100 Bp
DNA Ladder
PCR amplified product
210 Bp
Amplicon
B)
100 Bp
DNA Ladder
Purified & digested
Amplicon
Purified & digested
Vector
C)
1 Kb
DNA Ladder
Ecor I
XhoI
Ecor I / XhoI

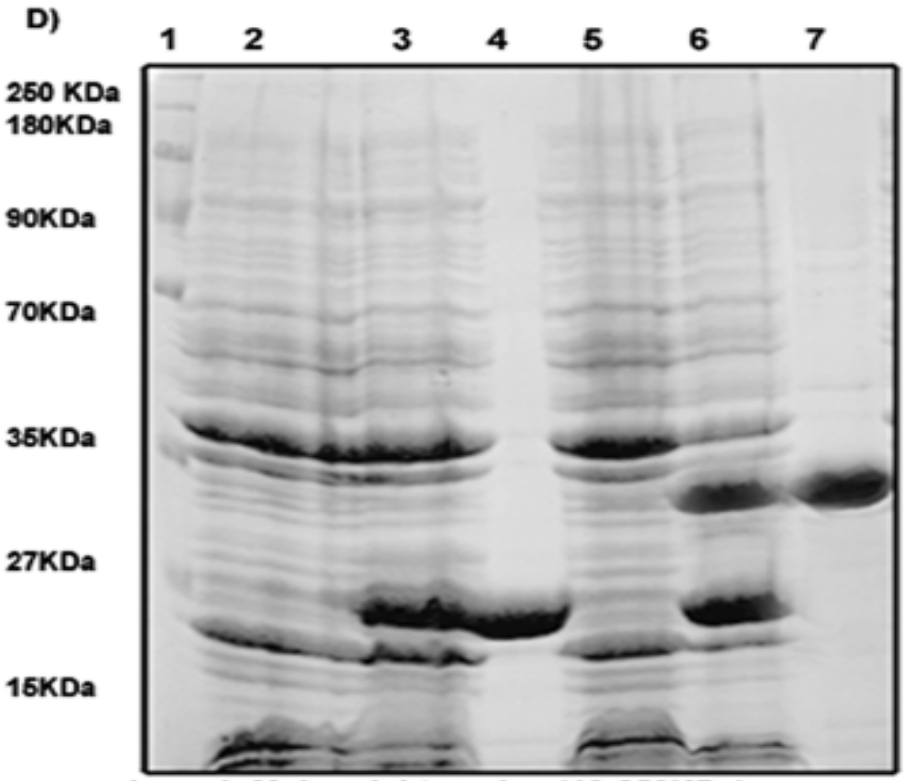


Figure 3.5: Cloning, expression and purification of GST-S6 protein (**A)** 1.5% Agarose gel showing PCR amplified 210 Bp fragment corresponding to carboxy terminal 69 amino acids of S6 protein **(B)** 1% Agarose gel showing restriction digestion of PGEX-4T2 vector and S6 insert **(C)** 1% Agarose gel showing cloning of S6 protein in frame with GST in PGEX-4T2 vector **(D)** 12% SDS-PAGE gel showing induction and purification of 33 kDa GST fusion protein (69 amino acids) and comparison of its molecular weight with GST.

Part II

S6 kinase α1 has been shown to coordinate various cellular processes involved in cell growth and proliferation in a phosphorylation dependent manner. Multiple independent phosphorylations have been proposed to account for complete activation of the enzyme with two phosphorylations one in the catalytic domain (T252) and other hydrophobic motif (HM) T412 in the linker domain, deemed to be most critical for enzyme activity. Incidentally, TOR kinase dependent HM phosphorylations (T412) has been implicated in rapamycin mediated inhibition, reflected by its selected de-phosphorylation attributed to direct inhibition of the TOR kinase. A series of phosphorylations in the carboxy terminal auto inhibitory domain (AID), marks the beginning of a sequence of ordered events to bring about eventual phosphorylation at activation loop (T252) and hydrophobic motif (T412) of the enzyme, identified in accordance with the homology of p70S6 Kinase with AGC subfamily of serine–threonine kinases. These catalytic domain phosphorylations help the enzyme assume a fully active conformation; the order in which the two sites are phosphorylated however, remains somewhat controversial. While PDK1 is the kinase responsible for activation loop phosphorylation, TOR kinase is believed to phosphorylate hydrophobic motif at T412. Conversely loss of phosphorylation at these sites in particular at T412 has been shown to inactivate the enzyme. The dynamics of these mitogen regulated phosphorylations has therefore, been the focal point of investigation to understand this complex regulation. The immunosuppressive agent rapamycin and its cellular receptor FKBP12, inhibit p70S6 kinase by causing selective loss of T412 phosphorylation, largely attributed to direct inhibition of TOR kinase although, recruitment/stimulation of phosphatase 2A also stands implicated in the process.

In addition to Insulin and other growth factor stimulation, p70S6 kinase has also been reported to get activated in response to viral infection, such that baculovirus mediated expression of the enzyme in insect cells, activates the enzyme by phosphorylation at similar sites as identified in the enzyme from regulated cells. Since it stands established that insect TOR-kinase, behaves similar to that of its mammalian counterpart, it has been foresighted that activation state and rapamycin inhibition

would be no different than the one established for mammalian systems. Furthermore, since the stimulus due to viral infection and the response of the enzyme can at no point be disengaged in Sf9 cells, the state of S6 kinase activation could be deemed as constitutive and therefore, ideal to investigate the dynamics of activating phosphorylations in presence of rapamycin. We therefore set out to identify rapamycin inhibitory mechanism and its phosphorylation dependence as well as studying the dynamics of critical phosphorylations (T412 & T252) that regulate enzyme in response to various inputs.

3.6 Comparative activity and rapamycin response of Baculovirus recombinant (BVr) and HEK S6K

Baculovirus recombinant (BVr) ribosomal protein S6 kinase (S6K1), expressed in Sf9 cells was active towards phosphorylating GST-S6 in conformity with earlier reports. The activity of the recombinant enzyme was 2-3 folds less than the random activity (without serum withdrawal or stimulation) exhibited by the enzyme transiently expressed in HEK 293 cells. Since the HEK enzyme could be activated a further 2-3 fold following stimulation, the BVr- enzyme in effect, was 4-6 fold less active than its mammalian counterpart. As seen in **Figure 3.6**, the BVr- enzyme was as sensitive to inhibition by rapamycin in a manner more or less comparable to the inhibition curve obtained for transiently expressed enzyme in HEK 293 cells. We have consistently observed slight recalcitrance of the enzyme to rapamycin inhibition, when the drug treatment is carried out without serum deprivation or after serum stimulation of the enzyme. Since serum or amino acid deprivation does not recreate the serum starved state in Sf9 cells, the concentration of rapamycin required to bring about inhibition was obviously higher than required otherwise. Furthermore the quantum of protein expression in Sf9 system was also an important determinant in establishing inhibitory concentration of the drug (not shown). Accordingly 20-24hr post infection period, with a multiplicity of infection (MOI) of $\leq$ 1 was chosen as optimal time point where the level of recombinant protein was appropriate to achieve > 90% inhibition in activity at a concentration of 50nM rapamycin.

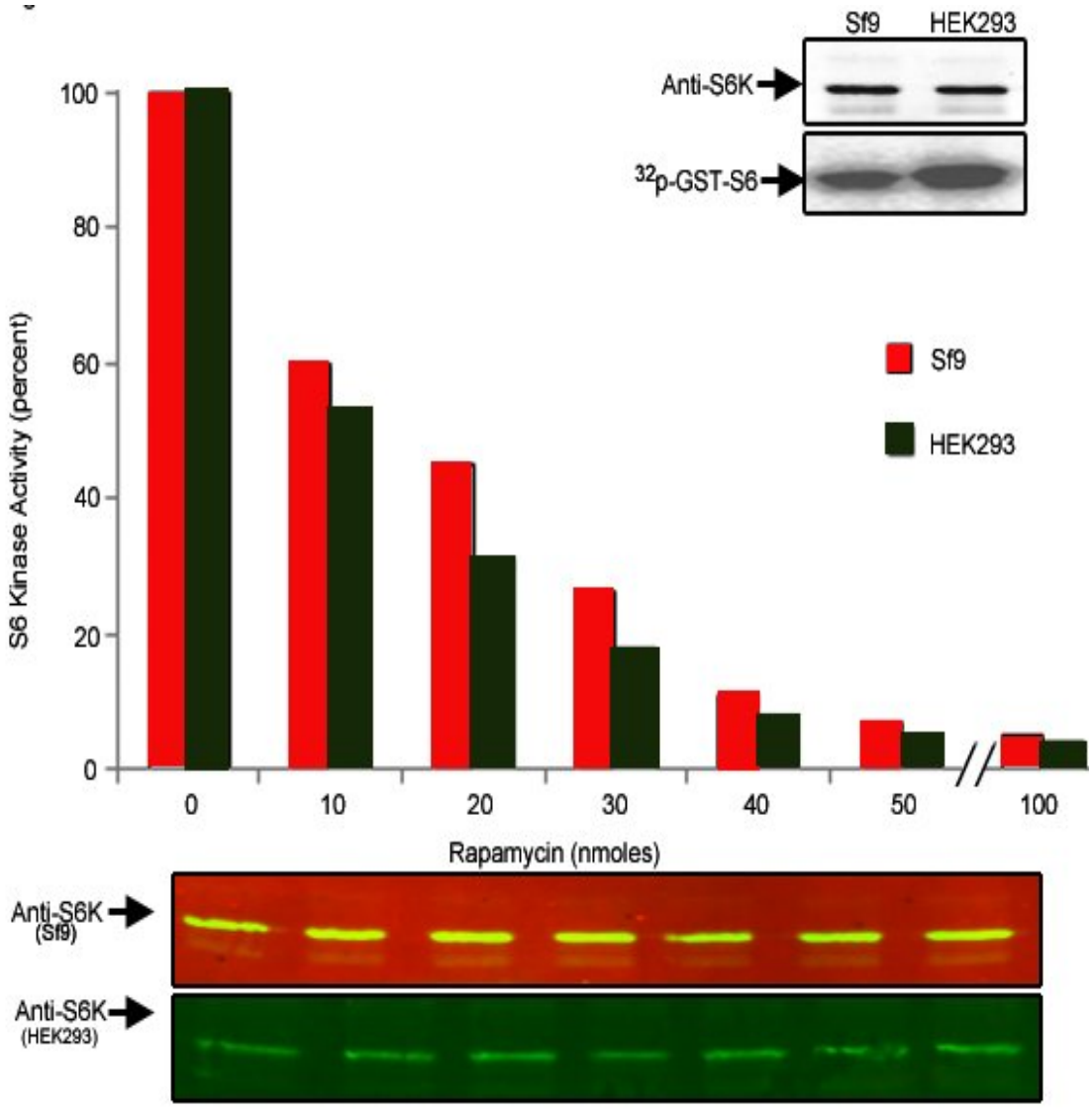


Figure 3.6: Comparative activity and rapamycin response of Baculovirus recombinant (BVr) S6K: Sf9 cells were infected with HA-S6K1α1 virus at an M.O.I of ≤1 for 24 hours and HEK-293 cells were transiently transfected with HA-S6K1 vector and grown for 48h in DMEM containing 10% FBS. Cells were exposed to rapamycin or ethanol for 15 min at concentrations indicated before harvest. Lysates were clarified, used for inmunoprecipitation with α HA antibody and immune complexes recovered were subjected to kinase assays using ^{32}pGSTS6 as a substrate (Inset) or S6 peptide. Samples were separated on a 12% SDS-page and transferred on to a PVDF membrane for autoradiography, immunoblotting, peptide kinase assays were performed by standard procedure and the activity in CPM normalized for comparison.

3.7 Status of activating phosphorylations in HEK and BVr S6K1

Immunoblot analysis using anti-phospho T412 and T252 antibodies easily established these phosphorylations in the enzyme immuno precipitated from HEK 293 cells, whose levels were seen to decrease considerably in the enzyme recovered from rapamycin treated cells **(Figure 3.7)**. Surprisingly the antibodies failed to identify any of these phosphorylations in the BVr- enzyme in a series of experiments, even when the membranes with commasie stained bands were probed. The absence of these phosphorylations in the BVr- enzyme though conceivable in view of its lesser activity was surprising to account for its continued inhibition by rapamycin in the context of substantial evidence implicating these phosphorylations, especially T412 to mediate the inhibitory effects of the drug .

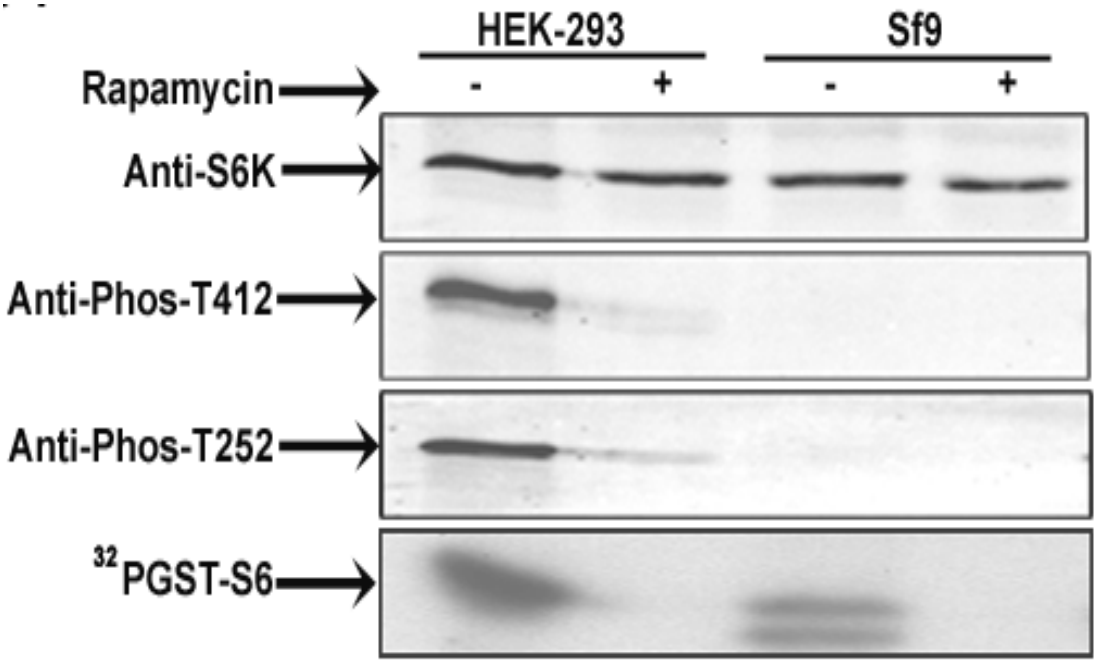


Figure 3.7: Status of activating phosphorylations in HEK-S6K1 and BVr S6K1: HA-S6Kα1 immuno-precipitated from HEK 293 cells or Sf9 cells treated with or without rapamycin (50ng/ml), was either subjected to immune-complex kinase assay or transferred to PVDF membrane for blotting with indicated antibodies.

3.8 BVr-S6K1 is resistant to phosphatase inactivation

As phospho specific (T412 and T252) antibodies failed to detect any signal in the enzyme immunoprecipitated from Sf9 cells, which could be attributed to the absence of their respective kinases in the system or their inactivation. It could however, be argued that the presence of only a minute fraction of phospho T412 and T252 in the BVr- enzyme might escape detection through immuno blotting. That being the case, the BVr- enzyme would tend to be more sensitive to inactivation by phosphatase than otherwise. **Figure 3.8a** shows that potato acid phosphatase or phosphatase 2A failed to bring about any significant inactivation of the enzyme at concentrations that were effective in de-phosphorylating T412 from the HEK-immunoprecipitated enzyme, thereby disregarding the argument about the possible existence of a minor fraction of phospho T412 and T252 in the BVr enzyme. It is pertinent to emphasize that only a few important phosphorylation sites that include T252, T412 and Ser394 (S394) remain critical for activity in backdrop of the data that loss of phosphorylation sites in the carboxy terminal auto inhibitory domain (AID) does not bring about any appreciable change in the activity of the enzyme. As such the resistance of the enzyme to phosphatase inactivation could only be explained if these sites were either absent or not accessible for phosphatase action. Since T412 and T252 are established post translational events, and the kinases that phosphorylate these sites identified the contention of their inaccessibility was certainly not plausible. The only other site that assumed significance in terms of its requirements for enzyme activity in this system, S394 believed to be co-translational understandably continued to resist phosphatase action. Interestingly significant residual activity continues to be detected in the enzyme expressed in CHO-IR and NIH-3T3 cells even after the phosphorylation at T412 was more or less completely removed by phosphatase treatment (**Figure 3.8b**) lending credence to the observed resistance of BVr enzyme to phosphatase inactivation.

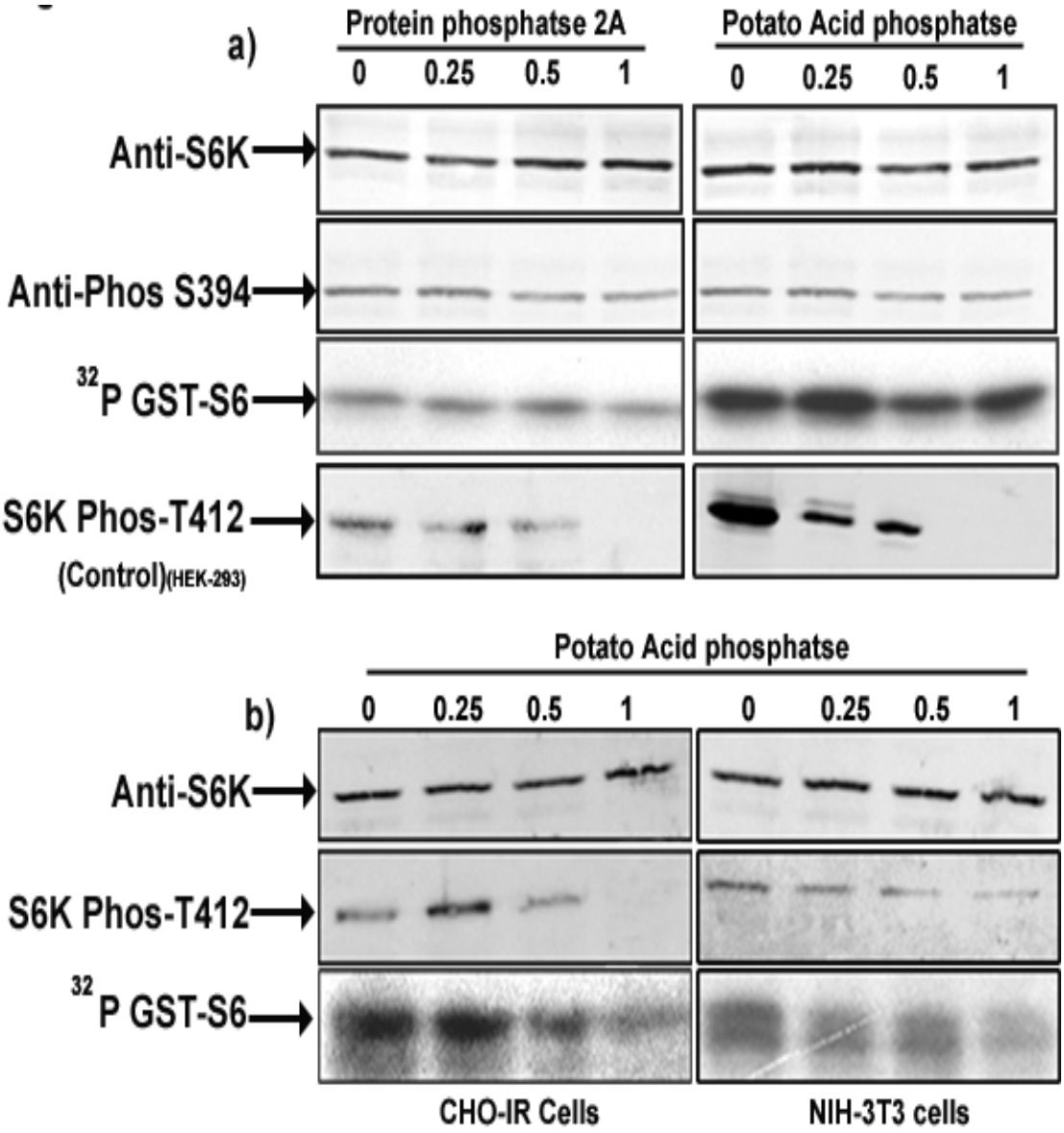


Figure 3.8: Phosphatase sensitivity of BVr- S6K1: S6Kα1-WT was immune-precipitated with anti HA antibodies from Sf9 cells infected with respective viruses as above. The immune-precipitates were split into four aliquots each and incubated with indicated concentrations of acid phosphatase (Figure 3.8a) or protein phosphates 2A (Figure 3.8b) for 30 min and washed with buffer containing phosphatase inhibitors and (5ηM) okadaic acid respectively, followed by a final wash with excess of kinase buffer, and processed as above.

3.9 TOR signalling input is not required for rapamycin inhibition of BVr enzyme and resultant phosphorylation at HM site is independent of TOR signalling motif

Since the activation and rapamycin sensitivity of the enzyme has also been shown to critically depend on the recruitment of TOR kinase through amino and carboxy terminal TOR signalling (TOS) motifs. It was imperative to examine, whether deletion of these motifs did indeed reproduce effects in accordance with the prevalent interpretations for mammalian cell system. S6K mutant truncated for amino terminal TOR signalling motif had activity comparable to WT S6K, and the same character was shown by the mutant truncated for carboxy terminal TOR signaling motif. Surprisingly the double mutant exhibited 2-3 fold more activity and partial resistance to rapamycin, in conformity with its reported behaviour in mammalian cells (**Figure 3.9**).

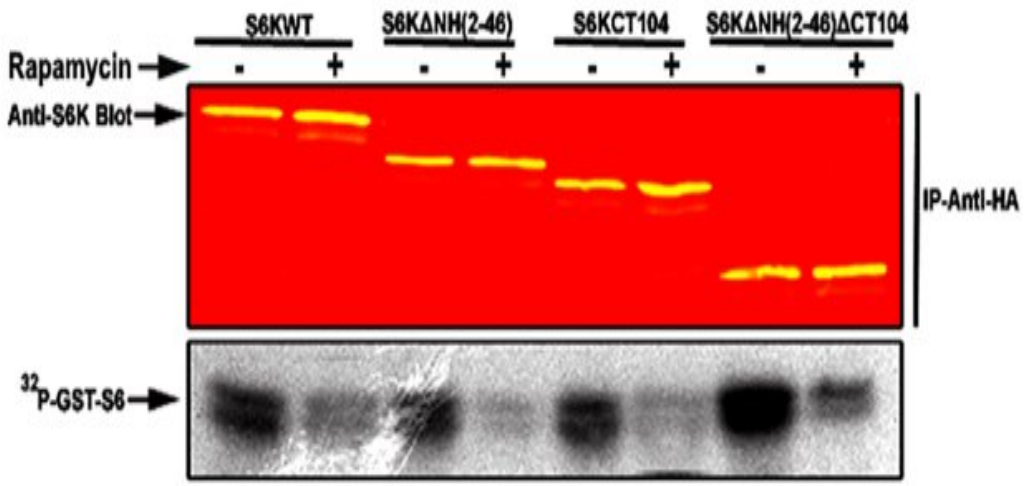


Figure 3.9: Rapamycin inhibition of Baculoviral enzyme is independent of TOR signalling input. (a) Sf9 cells were infected with recombinant viruses corresponding to S6KWT, S6K Δ2-46, S6KCT104, S6K (Δ2-46/ΔCT104) and cell lysates immuno-precipitated using anti HA antibody. Immune complexes were subjected to kinase assays and analysed by western blotting as described above. **(b)** Sf9 cells were infected with recombinant viruses corresponding to S6KWT, S6K Δ2-46, S6KCT104, S6K (Δ2-46/ΔCT104) and cell lysates immuno-precipitated using anti HA antibody. Immune complexes were subjected to kinase assays and analysed by western blotting as described above

However, the explanation attributing this mutation to facilitate direct phosphorylation at the HM is completely redundant in view of its absence in the BVr enzyme. It is therefore, safe to conclude that TOR recruitment and the resultant phosphorylation at the HM does not mediate the inhibitory effects of rapamycin.

3.10 HM and AL phosphorylations exhibit equal sensitivity to rapamycin inhibition:

We attempted to understand the contribution of each phosphorylation in mediating rapamycin sensitivity of S6K1 individually, and also as to how the turnover at each site relates to the turnover at the other. A time course analysis was set up to monitor the loss of HM and AL phosphorylations in response to rapamycin and relate it with S6K1 activity. HA-S6K1 transiently expressed in HEK 293 cells was immuno-precipitated following rapamycin exposure for different time points over a 10 min period and assayed for *in vitro* S6 phosphorylations. Kinase assays separated on SDS-PAGE were transferred on to a PVDF membrane, used for autoradiography followed by quantitative western analysis with fluorescently labelled antibodies. As seen in **Figure 3.10A**, loss of both HM and AL followed an identical time course without any obvious preference for one or the other phosphorylation, in contradiction with the data indicating preferential or selective loss of HM phosphorylation. Interestingly, more than 70% loss of S6K1 activity at 2.5 min of rapamycin exposure was associated with a modest (20-25%) loss of total fluorescent intensity for each antibody (**Figure 3.10B**). No significant loss in fluorescent intensity for either phosphorylation was observed over the next five min to end up with a residual fluorescence of 30-40% at 10 min, at which point the enzyme activity was lost completely. In other words, the loss of fluorescence or the change in mobility associated with either phosphorylation did not at any point appear to correlate with the loss of enzyme activity. Despite limitations in absolute quantitation, the data was compelling enough to indicate that rapamycin did not cause preferential dephosphorylation at either site to raise the possibility that loss of phosphorylations might be a co-ordinate event.

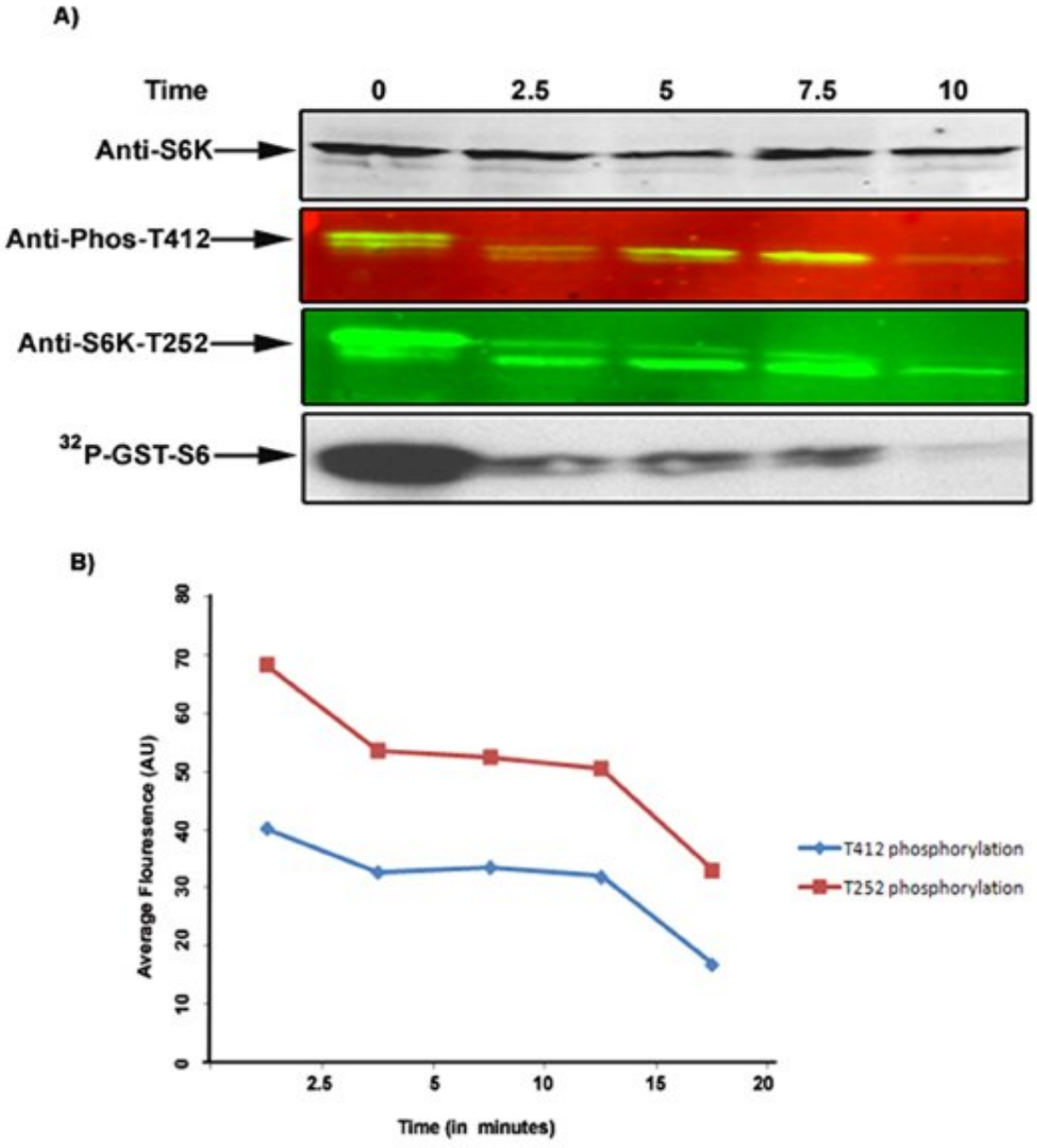


Figure 3.10: Equal sensitivity of HM and AL phosphorylations to rapamycin inhibition: (A) S6Kα1-WT was immunoprecipitated with anti HA antibodies from HEK-293 cells after treatment with 50nM rapamycin for different time intervals (As shown). The immune-precipitates were washed with lysis buffer containing 500mM NaCl followed by a final wash with excess of kinase buffer and processed as above **(B)** Fluorescence quantitation (in AU) of phosphorylations using LI-COR odyssey software.

3.11 HM and AL phosphorylations are individually resistant and co-ordinately sensitive to rapamycin inhibition.

To examine which if any one of the phosphorylations was more vulnerable to rapamycin inhibition, we introduced phospho-mimicking mutations at one of the sites to study the fate of the phosphorylation at the other. The mutants T252E and T412E, along with wild type S6K1 expressed in HEK 293 cells, were immuno-precipitated after serum starvation and or stimulation in presence or absence of rapamycin and subjected to immune complex kinase assays. The reaction mixtures were separated on SDS-PAGE gel and transferred on to a PVDF membrane which was first autoradiographed and later used for western blotting with various antibodies. **Figure 3.11** shows that WT S6K1 was stimulated 4-5 fold (compare lane 1 and 2) and completely inhibited by rapamycin (lane 3). T252E mutant exhibited very low activity (lane 4-6) for any judgment about its stimulation or its response to inhibition by rapamycin. T412E mutant on the other hand exhibited modest but constitutive activity with 60% resistance to inhibition by rapamycin (Lane7-9), largely in conformity with its reported behaviour. Immunoblot analysis using anti phospho-threonine 252 and 412 antibodies presented an interesting picture such that T252E engendered phosphorylation at T412 and T412E caused phosphorylation at T252. Though the extent of phosphorylation was quite modest (20-30%), it pointed towards the possibility of their interdependence. Surprisingly neither phosphorylation in the background of a mutation was sensitive to rapamycin, whereas both were effectively dephosphorylated in the wild type enzyme, to strongly suggest their interdependent or co-ordinate behaviour.

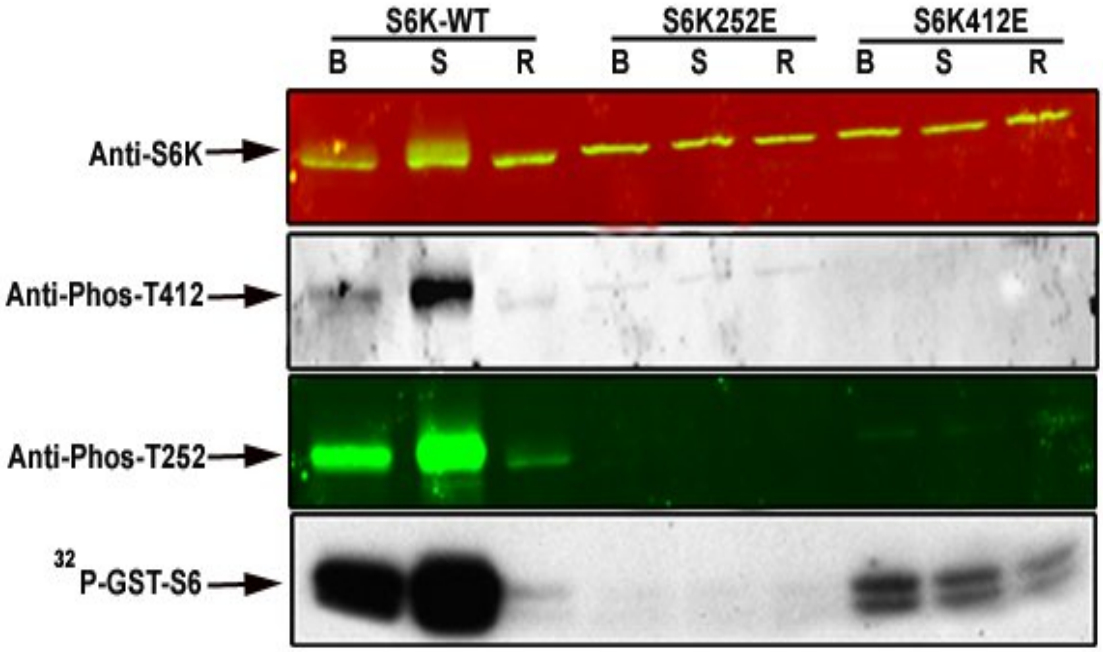


Figure 3.11: HM and AL phosphorylations exhibit individual resistance and co-ordinate sensitivity to rapamycin inhibition: HEK-293 cells were transfected with S6KWT, S6K252E and S6K412E were harvested 48h post transfection after treatment with rapamycin (50nM). Lysates were clarified, used for immunoprecipitation with α HA antibody and immune complexes recovered were subjected to kinase assays using ^{32}pGSTS6 as a substrate. Samples were separated on a 12% SDS-page and transferred on to a PVDF membrane for autoradiography. Alternatively, membranes were probed with indicated antibodies, and analysed using LI-COR infrared imager.

3.12 HM and AL phosphorylations exhibit conformation sensitive co-ordinate turnover in vivo

To further evaluate the turn over dynamics of HM and AL phosphorylations, we chose to use baculoviral expression system, wherein the wild type enzyme fails to ensue either of these phosphorylations, to minimize the cause and effect bias associated with such analysis in HEK 293 cells. As seen in Figure **3.12A**, introduction of T252E and T252A had no significant effect on the activity and rapamycin sensitivity compared to WT enzyme, in conformity with the data that T252E does not reproduce phospho mimicking due to steric sensitivity associated with AL site.

Phospho deficiency due to T252A used as a control was expectedly redundant in influencing enzyme activity because of the absence of this phosphorylation in the first place. Surprisingly however, both T252E and 252A mutations caused the enzyme to get phosphorylated at T412. Even more surprising was the fact that T412 phosphorylation was quantitatively similar in presence and absence of rapamycin, despite complete inhibition of enzyme activity, substantiating the observation made in HEK-293 cells above. The observation implied that kinase mediating T412 phosphorylation was not inhibited by rapamycin in complete contradiction with the dogma implicating rapamycin sensitive TOR-kinase in mediating this phosphorylation. To further investigate whether T412 phosphorylation was in anyway dependent on TOR-input, F28A mutation that abolishes TOR kinase recruitment was introduced alone and in the background of T252E mutation. As seen in **Figure 3.12B**, T412 phosphorylation remained unaffected in the double mutant (F28A-252E) to indicate that the kinase that phosphorylates T412 *in vivo* was oblivious to TOR signalling input. T412E and T412A on the other hand produced dramatic effects both on the activity and rapamycin sensitivity, exhibiting 6-8 folds and 2-3 folds increase in activity in comparison to wild type enzyme respectively. While T412E showed near complete resistance (>80%) to rapamycin inhibition, T412A was 10-20% less sensitive than the WT enzyme. Immunoblotting with anti-phospho T252 antibodies revealed prominent presence of T252 phosphorylation in T412E mutant and the phosphorylation in T412A mutant though present was barely detectable. These data indicate that intervention at either site governs the turnover at the other site characteristic of a co-ordinate event.

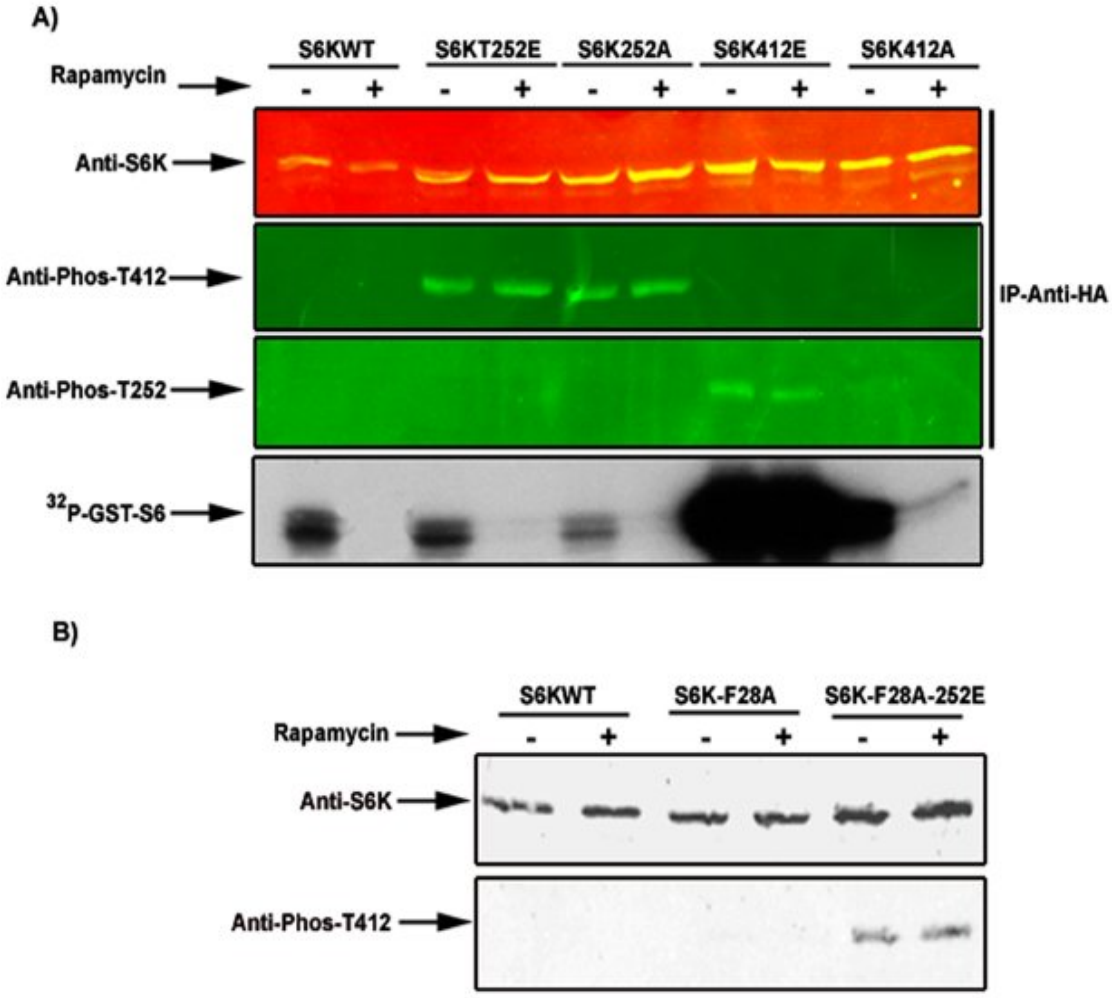


Figure 3.12: Activation and rapamycin sensitivity are mediated by coordinate turnover of phosphorylations at AL and HM: (A) Sf9 cells were infected with recombinant viruses corresponding to S6KWT, S6K 252A, S6K252E, S6K 412E, and S6K412A at an M.O.I of ≤1 for 48 hours. Cells were exposed to rapamycin or ethanol for 15 min at concentrations of 50nM before harvest. Lysates were clarified, used for immunoprecipitation with α HA antibody and immune complexes recovered were subjected to kinase assays using ^{32}pGST-S6. Samples were separated on a 12% SDS-page and transferred on to a PVDF membrane for autoradiography, immunoblotting, peptide kinase assays were performed as described.

(B) Sf9 cells were infected with recombinant viruses corresponding to S6KWT, S6K F28A, and S6K-F28A-252E at an M.O.I of ≤1 for 48 hours. Cell lysates were immuno-precipitated using anti HA antibody. Immune complexes were subjected to kinase assays and analysed by western blotting as described above.

3.13 HM and AL phosphorylations do not exhibit a preferential sequence of occurrence *in vitro*

To further substantiate that the two phosphorylations did indeed not prefer a particular sequence of occurrence, T252E and T412E mutants expressed in insect cells were immunoprecipitated and treated with potato acid phosphatase to remove the endogenous phosphorylations at T412 and T252 respectively. Phosphatase treated T252E and T412E mutants were then incubated with HEK 293 extracts prepared from cells treated with or without rapamycin. **Figure 3.13 (a)** shows that enzymes present in HEK extracts phosphorylated the mutant enzymes without any obvious preference for one or the other background mutation. Interestingly the extracts from rapamycin treated cells phosphorylated the mutants as effectively as those from untreated cells to substantiate the observation that *in vivo* kinases responsible for phosphorylation of both sites were insensitive to rapamycin as was observed in the insect cells above**.** To provide further credence to the argument, S6K was co-expressed with constitutively active mTOR and PDPK1 in Sf9 cells using baculoviral infection **(Figure 3.13 b)**. S6K was found to be phosphorylated at both HM and AL when both kinases (mTOR and PDPK1) were individually co-expressed with WT S6 kinase, suggesting that phosphorylation at either of the two sites leads to phosphorylation at the other site. This data clearly implicates that both T412 and T252 are regulated in a coordinate manner and phosphorylation at either site primes the enzyme to get phosphorylated by upstream kinases at the other site.

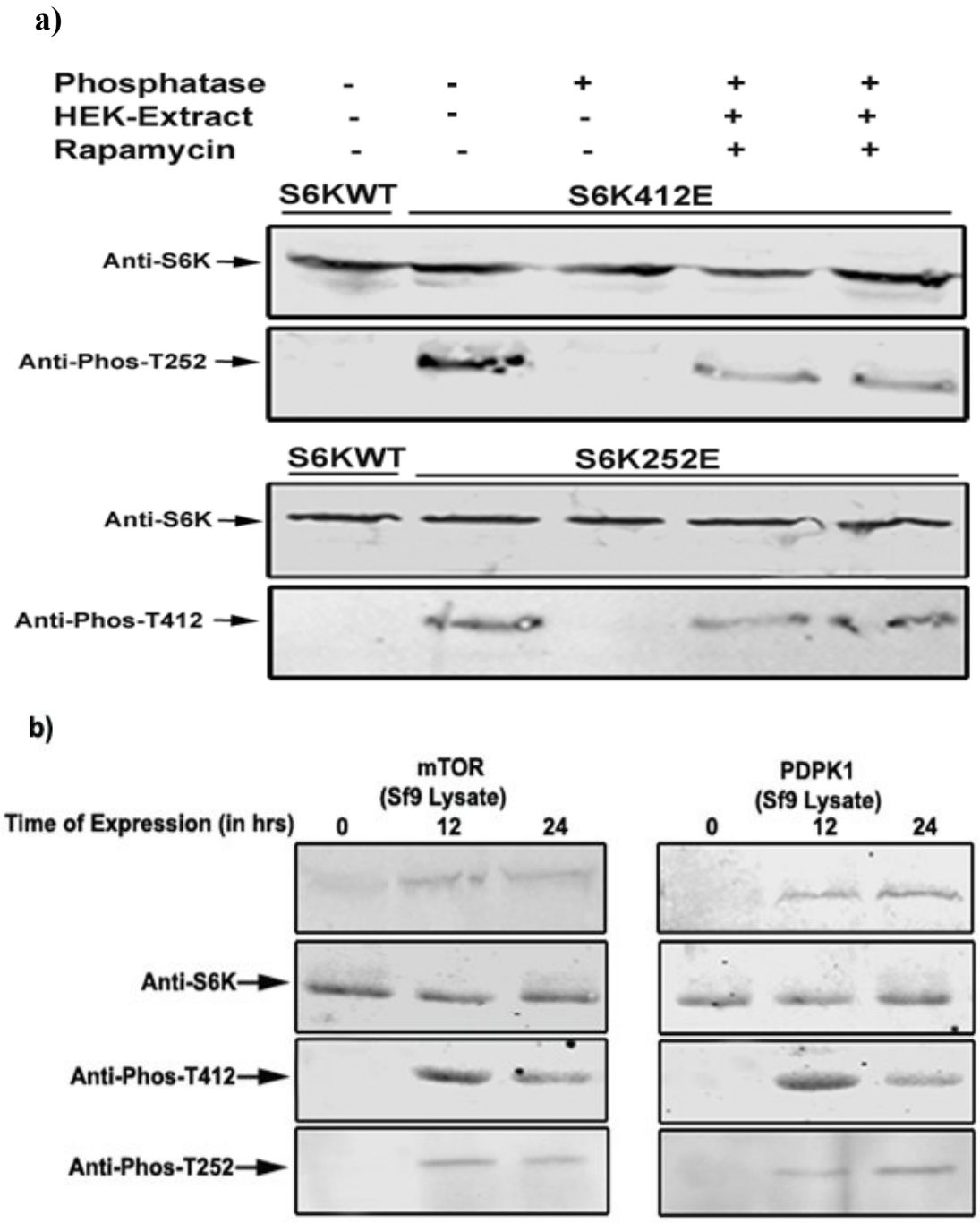


Figure 3.13: HM and AL phosphorylations do not exhibit a preferential sequence of occurrence *in vitro*: a) S6KT252E (Panel I) and S6KT412E (Panel II) mutants were expressed in insect cells, immuno precipitated and treated with potato acid phosphatase to remove the endogenous phosphorylations at T412 and T252. An overlay assay with HEK-293 extracts (with or without rapamycin) in presence of $MgCl_2$ was done, and processed for western blotting with different antibodies as above. **b)** S6KWT was co-expressed with constutively active mTOR and PDPK1 in Sf9 cells by Baculoviral infection, immunoprecipitated and probed with phospho specific antibodies as above.

3.14 Conformational state and not phosphorylations *per se* governs the sensitivity of S6K1 to rapamycin

The insect cell data above (**Figure 3.12A**) indicated that introduction of Alanine at either site was surprisingly close in reproducing the effects of glutamate at these sites, suggested that the conformation achieved by the enzyme phosphorylated at both AL and HM, can perhaps be approximated by flexible alanines.

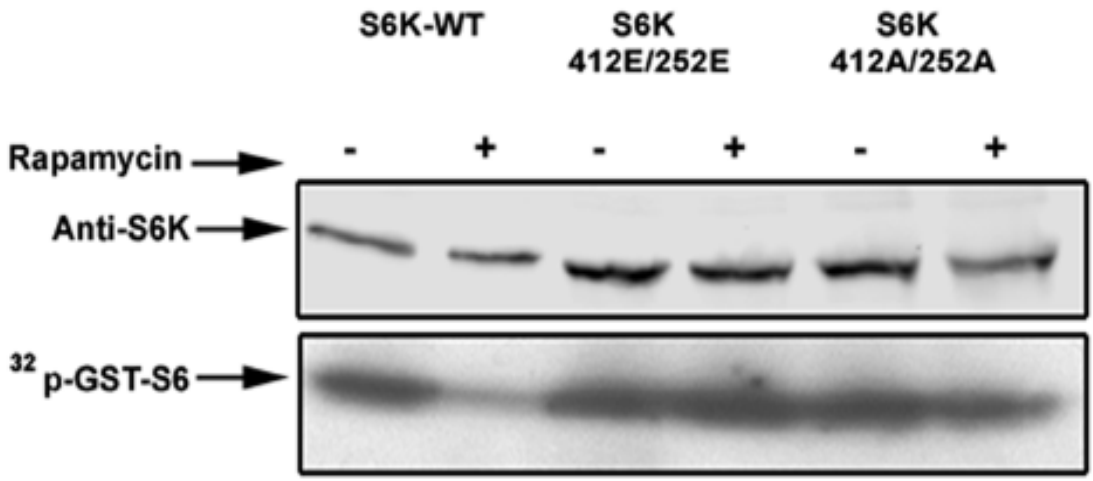


Figure 3.14: Conformational state and not phosphorylations *per se* governs the sensitivity of S6K1 to rapamycin: S6KWT, double mutants S6K T252A/T412A, S6K T252/T412E were expressed in Sf9 cells by Baculoviral infection, immunoprecipitated and kinase assay using ^{32}pGST-S6 was performed and western blotting done as above.

Double mutants with AL and HM threonines mutated to alanine (T252A, T412A) or glutamate (T252E, T412E) were thus generated for comparison of their sensitivity to rapamycin. **Figure 3.14** show that while the activity of either mutant was no different than the WT enzyme, both mutants exhibited significant resistance to rapamycin inhibition. While double E mutant was completely resistant, the double A mutant exhibited more than 60% resistance to rapamycin to indicate that conformational state and not the negative charge at each site dictates the status of rapamycin sensitivity.

3.15 Proposed model of S6K activation

Prevailing model for S6K1 regulation associates a central role with dynamics of sequential phosphorylations at the HM and AL of the enzyme, such that the HM phosphorylation supposedly brought about by TOR-kinase, primes the enzyme for PDK1 dependent phosphorylation at the AL for its full activation. Accordingly loss of HM phosphorylation attributed to TOR-kinase inhibition, with resultant loss of AL phosphorylation is the hypothesis put forward to explain the mechanism of rapamycin inhibition. Accordingly we propose a new model that better describes the picture and leads to better interpretation of earlier data. The clue to explain the model for the loss of two phosphorylations in response to rapamycin (**Figure 3.15**) comes from the behaviour of phospho deficient mutants T252A and T412A, such that their introduction paradoxically induces changes approximating those observed for phospho mimicking variants, to perhaps suggest that a conformational change is necessary to engender the two phosphorylations which is somehow incapacitated in the insect cell system. This conformational change perhaps brought about by release of an inhibitor upon stimulation must result in simultaneous phosphorylations at AL and HM for full activation of the enzyme. Locking the enzyme in the active conformation can be achieved by preventing turnover of phosphorylation at either site. Rapamycin in collaboration with TOR must work to potentiate binding of the inhibitor to the enzyme to propagate inhibitory conformation that results in consequential loss of the two phosphorylations.

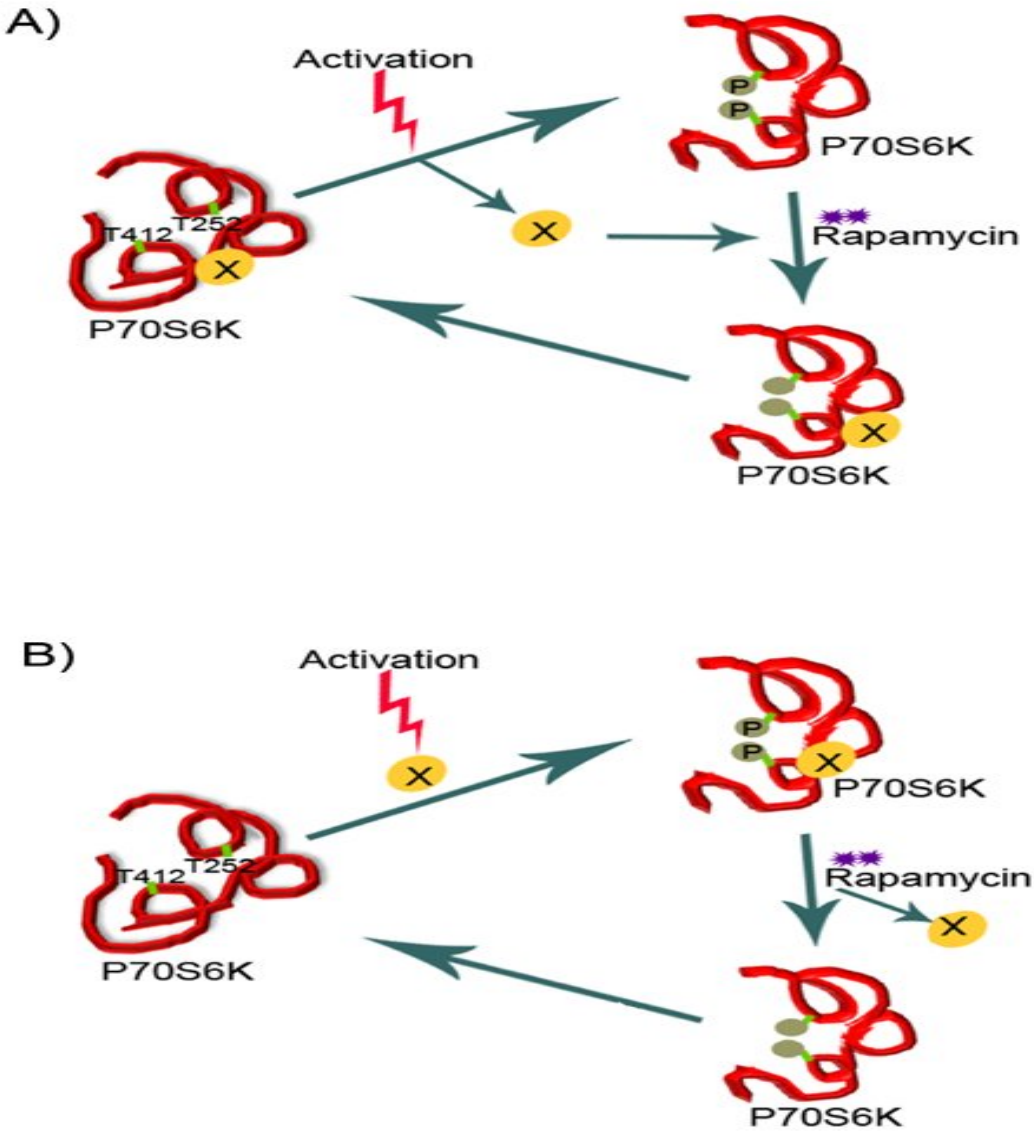


Figure 3.15: Model depicting the proposed mechanism of S6K1 activation and its inhibition by rapamycin: A) An unidentified regulatory factor (X) remains bound to S6K1 in its inactive state such that HM and AL sites are not accessible for phosphorylation by their respective kinases. The stoichiometry of its binding will dictate the number of enzyme molecules available for activation. Growth factor stimulation dislodges the factor to facilitate co-ordinate phosphorylation at AL and HM. Rapamycin potentiates binding of the factor to the enzyme, to render the phosphorylation sites inaccessible after their consequential loss. **B)** The cellular factor binds as a result of enzyme activation causing phosphorylations at HM and AL and is dislodged on rapamycin treatment of the cells leading to consequential loss of phosphorylations at HM and AL.

Part III

In order to find out possible influence of AKT and MAPK pathway on the status of S6 kinase, a drug based approach wherein the influence of these pathways on S6K could be established was desired. Accordingly, we used two amino amide anaesthetics bupivacaine and lidocaine which are known to inhibit AKT and MAPK pathway in several cell lines mediating at least some of the toxicity attributed to the use of these drugs. Although inhibition of ERK and AKT pathways seemingly appear to mediate some of the effects associated with their use, their long established inhibitory influence on amino acid sensing hints at possible involvement of S6 kinase pathway in mediating some of the associated effects. We accordingly attempted to relate the growth inhibitory effects of these LAs with the status of S6K1 in a way to establish molecular cross talk between the two pathways.

3.16 Cellular response to LAs is cell type specific

The cytotoxic effects of local anaesthetic drugs relate to the physiological response of individual cell type. Accordingly cells exhibit differential sensitivity to toxicity of these agents. In order to examine the cellular changes mediating such effects, it was necessary to identify a cell type that was optimally suited for the study without undue interference from global cessation of cellular processes. A series of cell lines were thus examined for their sensitivity to varied concentrations of LAs as monitored by MTT assay. As shown in **Figure 3.16**, the C6 neuroglial cells were maximally sensitive to the toxic effects of LAs with more than 80% cell death within hours of LA exposure. Fibroblasts (NIH-3T3) on the other hand exhibited considerable resistance to cell death in comparison to other cell types with an IC_{50} value of 0.614 mM for bupivacaine and 1.44 mM for lidocaine. An intermediate response was observed for a series of cell lines tested including C2C12 myoblasts which were thus excluded. Notwithstanding the in vivo scenario, since the IC_{50} of the drugs for NIH-3T3 fibroblasts being considerably less than the clinically applicable concentrations, cell line was deemed appropriate to provide a reasonable assessment of the mediatory events.

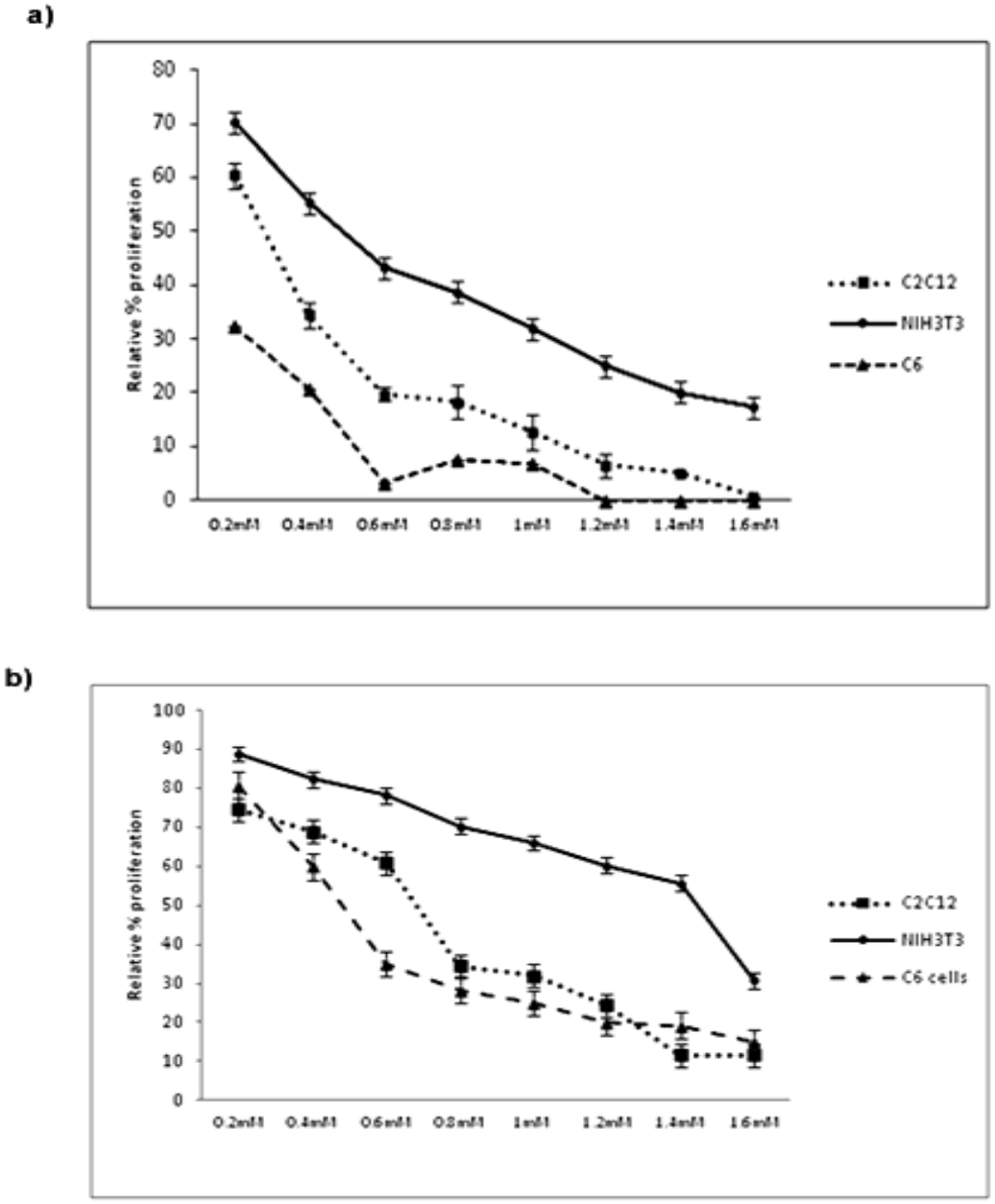
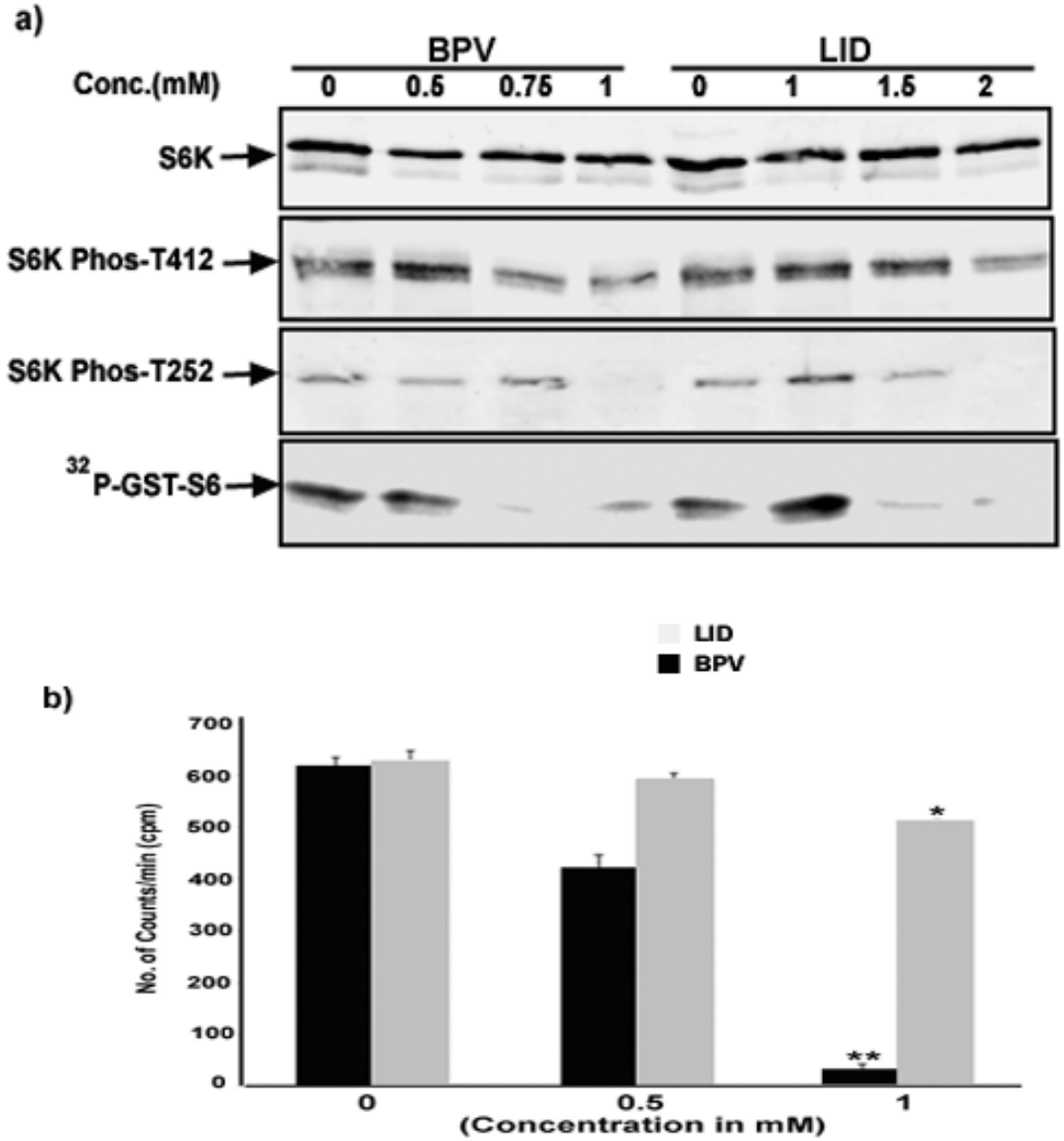


Figure 3.16: Dose-dependent inhibition of cell growth. NIH-3T3,C2C12 and C6 neuralgia cells were plated at a cell density of 5000 cells/well in triplicates under standard growth conditions (96 well format) for 24 hrs and incubated with indicated concentrations of bupivacaine **(a)** and lidocaine **(b)**.Cells were washed with cold PBS and subjected to MTT assays. Data are mean ±SEM with n=3 *vs.* control.

3.17 LAs inhibit S6K1 in a concentration dependent manner

Considerable body of evidence is suggestive of a possible involvement of S6 kinase pathway at least for some of the effects associated with LAs like apoptosis and growth inhibition, wherein the role of this pathway appears overwhelming. We therefore, sought to investigate any such possibility by assessing the activity status of S6K1 in presence or absence of the LAs. Accordingly ectopically expressed HA-tagged S6 kinase in NIH-3T3 cells grown in presence or absence of LAs was examined for its ability to phosphorylate GST-S6. As seen in **Figure 3.17a**, both bupivacaine and lidocaine inhibited the enzyme in concentration dependent manner.



While 0.65± 0.06 mM bupivacaine completely abolished S6 kinase activity, 2-3 folds more lidocaine was required to bring about comparable inhibition of the enzyme. The spectrum of concentration for both LAs was uniformly distributed to reside in the proximity of IC_{50} values for individual drugs. Minimal inhibitory concentration of each drug was then used to establish the time course of S6K inhibition. **Figure 13.17c** shows that complete inhibition of the enzyme activity by bupivacaine was achieved at 12 hours,

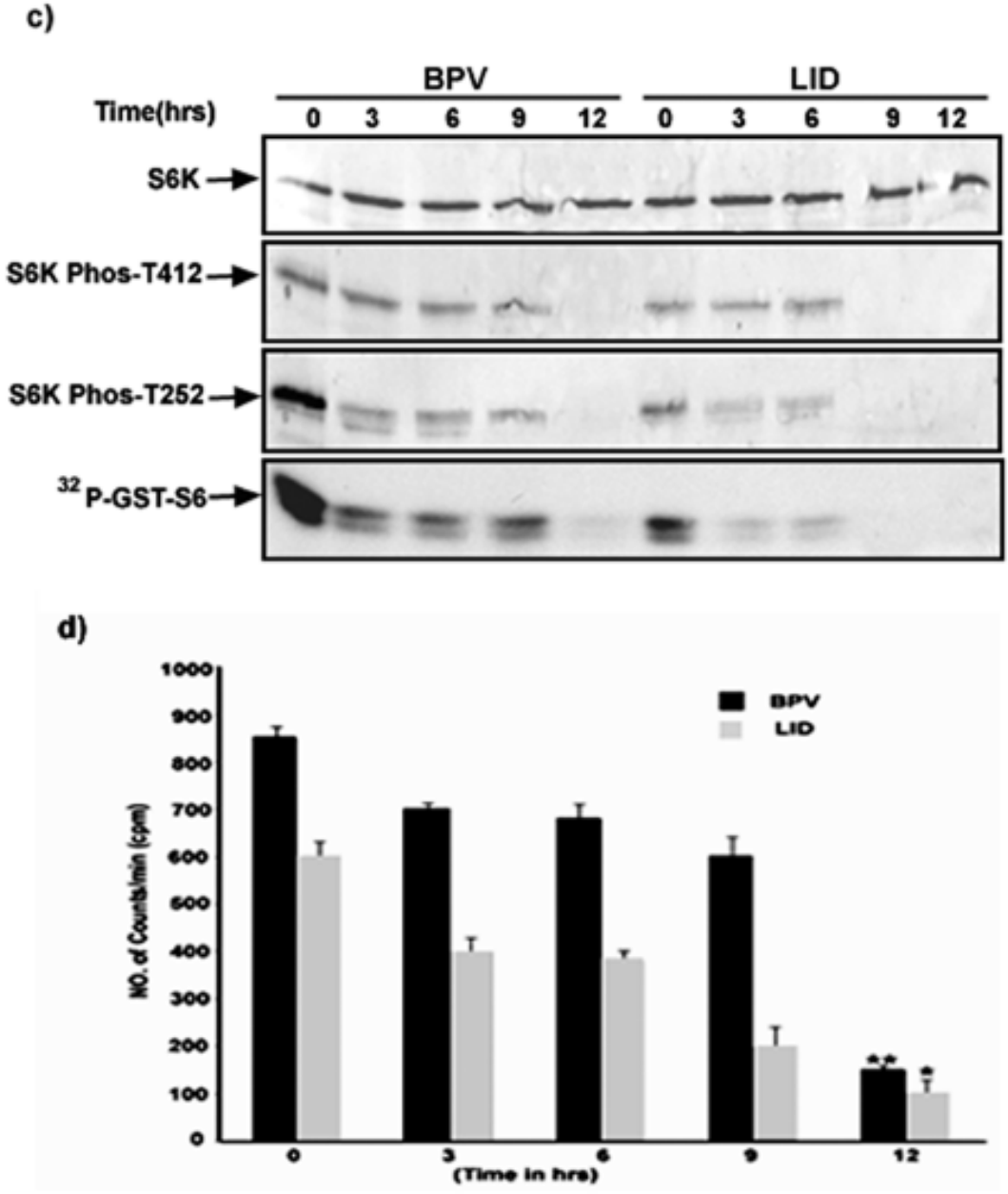


Figure 3.17: Concentration and time dependence of S6K1 inhibition by bupivacaine and lidocaine. a) NIH-3T3 cells transfected with pMT2-S6K1 were allowed to grow for 48 hrs grown in the absence (control) or presence of indicated concentrations of bupivacaine (BPV) or lidocaine (LID) for 12 h and immunoprecipitated S6K1 was subjected to kinase assays **b)** Quantification of S6K1 activity at different concentrations of bupivacaine and lidocaine from three independent experiments (lower panel). Data are expressed in mean ±SEM **/* (n=3) indicates P<0.05. **(c)** NIH-3T3 cells were transfected as above and grown in absence or presence of Bupivacaine (1mM) and Lidocaine (2mM) for indicated time intervals and processed similarly as above **d)** Quantitative analysis of S6K1 activity (lower panel). Data are expressed in mean ±SEM **/* for three independent experiments indicates P<0.05.

whereas lidocaine inhibited the enzyme at 6 h post treatment, indicating that two drugs exhibited independent inhibitory kinetics both with regard to concentration and time.

3.18 LA induced S6K1 inhibition is associated with loss of activating phosphorylations

Catalytic domain phosphorylations at the hydrophobic motif (HM) and activation loop (AL) of S6K1 are established determinants for the activity of the enzyme, such that enzyme inhibition is associated with loss of these phosphorylations. We therefore sought to ascertain whether LAs induced inhibition of S6K1 activity did indeed correspond with loss of these phosphorylations. As seen in **Figure 3.17 (a and c),** the inhibition of S6K1 correlated with concentration dependent loss of both T412 and T252 phosphorylations. In order to rule out the possibility that the observed loss of S6K1 activity was not due to contaminant phosphatase pronouncing its influence due to associated effects, an in-cell western assay using phospho specific antibodies

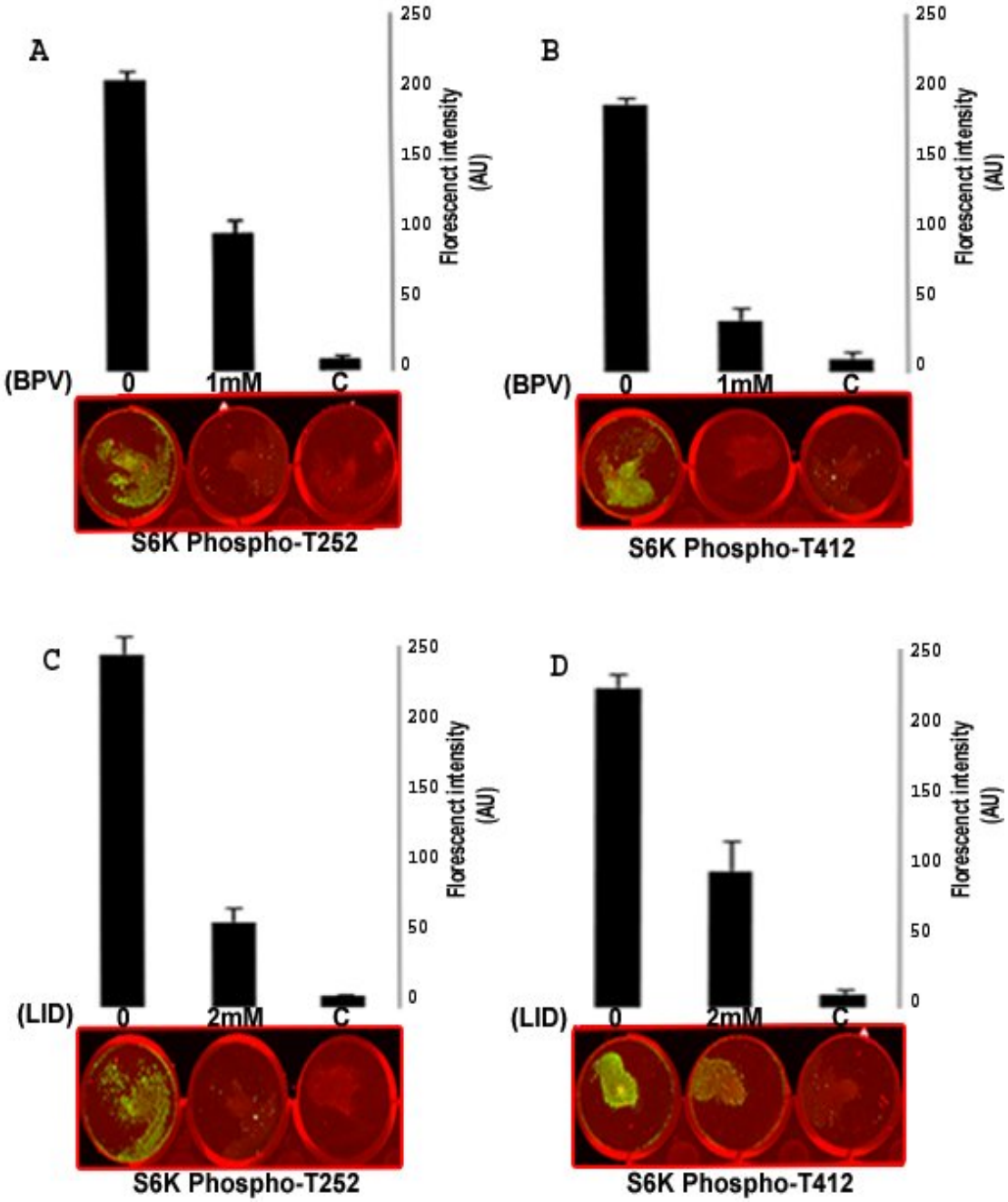
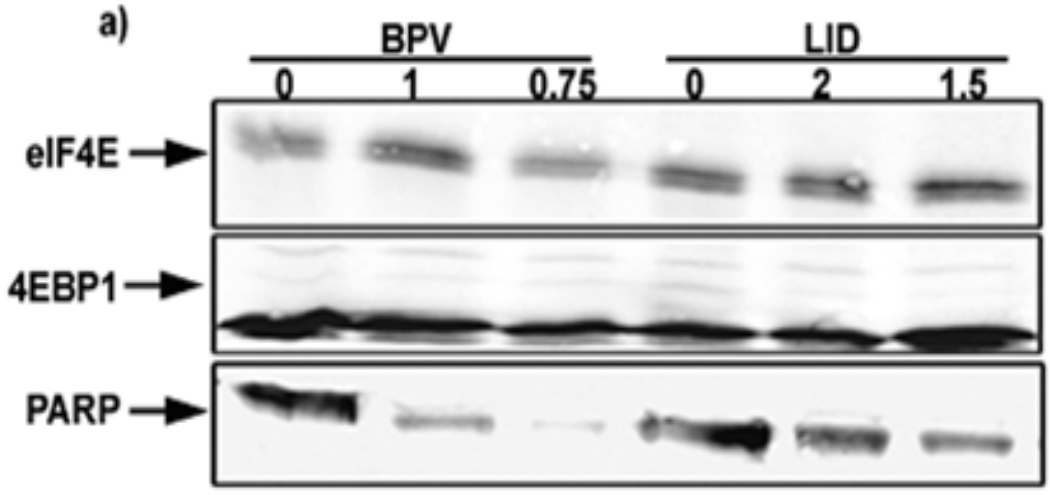


Figure 3.18: In-cell western assay showing loss of critical phosphorylations (T412 & T252): NIH-3T3 cells were transfected with HA-S6K1 and incubated for 48hrs before treatment with inhibitory concentrations of bupivacaine and lidocaine (1mM & 2mM respectively) as described above. Cells were processed for treatment with S6K Phospho-T412 and S6K phospho-T252 antibodies and imaged using LI-COR infrared imager. Average florescent intensity of each well was calculated in arbitrary units (AU) using LI-COR-ODYSSEY software.

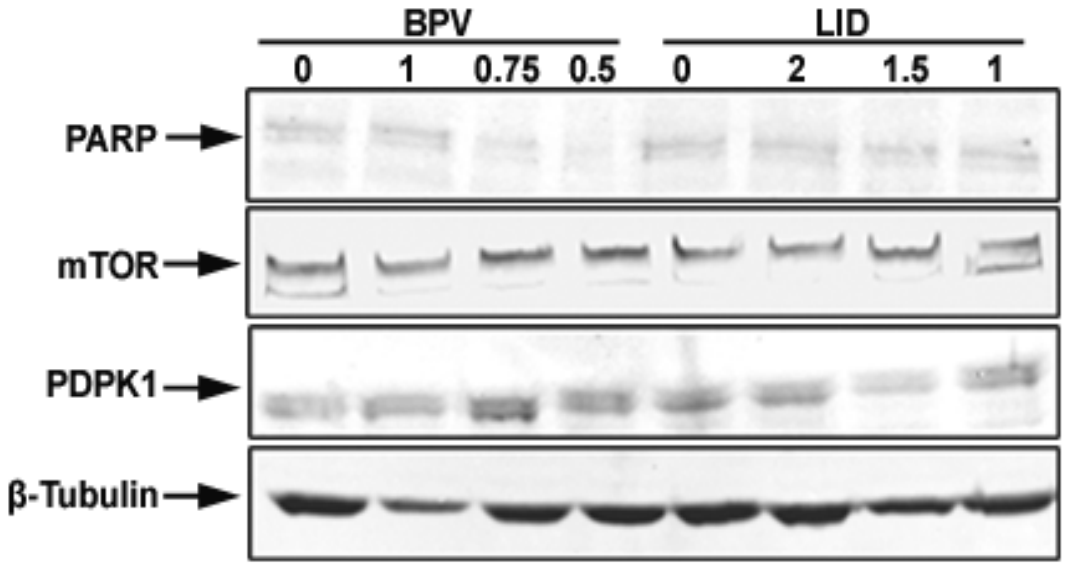
against T412 and T252 was performed. As seen in **Figure 13.18**, the relative fluorescent intensity for the individual phospho-antibodies exhibited similar pattern of disappearance in response to LAs treatment as observed for the immunoprecipitated enzyme

3.19 mTOR pathway does not mediate S6K inhibition by LAs

mTOR is considered to be an upstream activator of S6K1 and implicated in T412 phosphorylation of the enzyme and parallel phosphorylations of the other substrate eukaryotic translation initiation factor 4E binding protein 1 (4EBP1). Possible inactivation of mTOR pathway as implied by inhibition of S6K1 should therefore be corroborated by loss of such mTOR dependent phosphorylations in 4EBP1 and consequent loss of binding with translation initiation factor 4E. However, no detectable shift in mobility of various phosphorylated species of 4EBP1 was observed in presence of both LAs (**Figure 3.19a**). Furthermore binding of 4E known to be influenced by mTOR dependent phosphorylations of 4EBP1 continues to remain unchanged in the presence of either drug suggesting that mTOR pathway remained fully functional when compared to controls. To completely rule out any mTOR input for S6K inhibition mediated by LAs, an immuno-blot analysis of mTOR and other members of the pathway (**Figure 3.19b**) indicated that LAs did not bring about any significant change in their expression compared to poly ADP ribose polymerase (PARP) which is known to get down regulated in response to LA treatment, and thus acted as a positive control, suggesting that the selective inhibition of S6K was in no way related to change in expression characteristics of the mTOR pathway members.



b)


BPV
LID
0
1
0.75
0.5
0
2
1.5
1
PARP
mTOR
PDPK1
β-Tubulin

c)

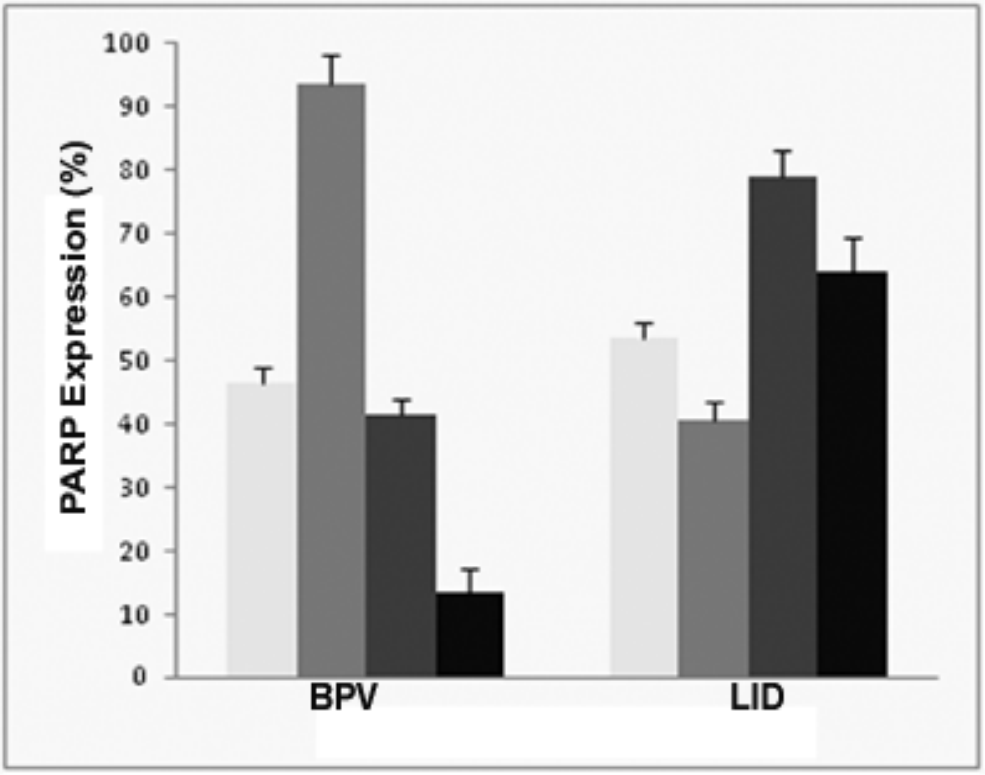

PARP Expression (%)
100
90
80
70
60
50
40
30
20
10
0
BPV
LID

Figure 3.19: LA mediated inhibition is independent of mTOR input: a) NIH-3T3 cells transfected with Myc-4EBP1 incubated for 48 hrs before treatment with indicated concentrations of bupivacaine and lidocaine. Lysates were clarified, used for immuno-precipitation with anti-Myc antibody on 7-methy GTP-Sepharose and immune complexes recovered were separated on an 18% SDS-PAGE gel transferred on PVDF membrane immuno-blotted with anti-4EBP1 and anti-eIF4E antibody. **b)** NIH-3T3 (untransfected) cells grown in the absence (control) or presence of bupivacaine (BPV) or lidocaine (LID) for 12 h and subjected to immuno-blotting with anti-mTOR, anti-PDPK1, anti-PARP and anti β-Tubulin antibodies respectively **c)** Expression analysis of β-Tubulin relative to PARP (positive control) using fluorescence based quantitation by LI-COR-ODYSSEY software.

Expression of PARP (positive control) was compared to loading control **(Figure 3.19 c)** using fluorescence based quantitation using LICOR odyssey software to show that PARP gets down regulated on LA treatment when compared to β-tubulin.

3.20 Bupivacaine and not lidocaine inhibits p90RSK1

The ability of bupivacaine and lidocaine to influence p90RSK was examined to establish specificity of LA inhibition and relate it with the reported inhibition of ERK. We show that (**Figure 3.20**) this kinase was inhibited by bupivacaine whereas lidocaine failed to show any effect at concentrations that inhibit S6K1. Higher concentrations of lidocaine could not however be tested due to massive cell death.

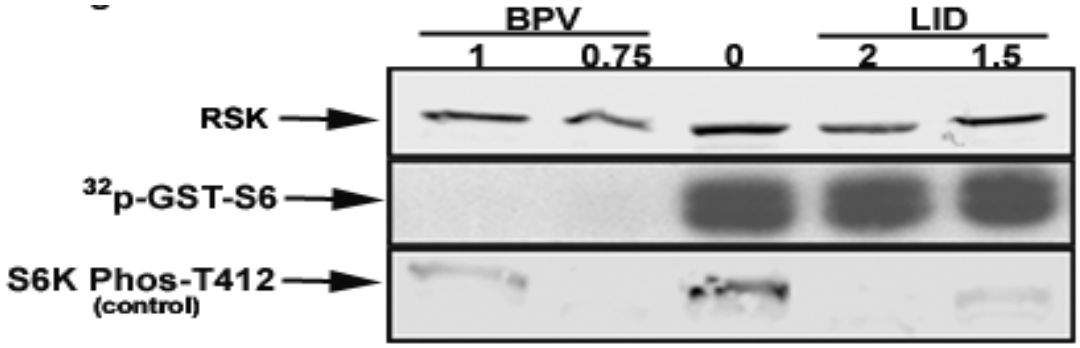


Figure 3.20: Bupivacaine and not Lidocaine inhibits activity of ERK substrate p90-RSK1: NIH-3T3 cells were transfected with HA-RSK1 and treated with indicated concentrations of bupivacaine and lidocaine as above. Lysates were processed for kinase assays as above using ^{32}pGST-S6 as a substrate, transferred on to a PVDF membrane, autoradiographed and probed with anti-RSK1 antibody.

Discussion Part I

Baculoviral expression of S6K1 in insect cells upon analysis indicated that contrary to common belief, the enzyme was only modestly active (4-6 folds less) than its fully active mammalian counterpart. The state of activity associated with the insect cell enzyme corresponded with the absence of both HM and AL phosphorylations. The enzyme surprisingly continued to exhibit sensitivity to rapamycin suggesting a possible redundancy of these phosphorylations in mediating rapamycin inhibition.

Although higher concentrations of rapamycin were required to bring about inhibition of the insect cell enzyme, it appeared in tune with the recalcitrance to rapamycin inhibition shown by the mammalian enzyme, when the drug treatment is carried out without serum deprivation or after serum stimulation. Since serum or amino acid deprivation cannot recreate a serum starved state in insect cells, the concentration of rapamycin required to bring about inhibition would obviously be higher than required otherwise. Furthermore the quantum of protein expression in Sf9 system was also an important determinant in establishing inhibitory concentration of the drug. Failure of phospho specific antibodies to detect any signal in the BVr- enzyme in a series of experiments, even on membranes with commasie stained bands was surprising in the context of substantial evidence implicating these phosphorylations, especially T412 to mediate the inhibitory effects of the drug. It could however, be argued that the presence of only a minute fraction of phospho T412 and T252 in the BVr- enzyme might escape detection through immuno blotting. That being the case, the BVr- enzyme would tend to be more sensitive to inactivation by phosphatase than otherwise. Potato acid phosphatase or phosphatase 2A however, failed to bring about any significant inactivation of the enzyme at concentrations that were effective in de-phosphorylating T412 from the HEK-293,CHO and NIH-3T3 immunoprecipitated enzyme, thereby disregarding the argument about the possible existence of a minor fraction of phospho T412 and T252 in the BVr enzyme. It is pertinent to emphasize that only a few important phosphorylation sites that include T252, T412 and Ser394 (S394) remain critical for activity in backdrop of the data that loss of phosphorylation sites in the carboxy terminal auto inhibitory domain (AID) does not bring about any appreciable change in the activity of the enzyme. As such the resistance of the

enzyme to phosphatase inactivation could only be explained if these sites were either absent or inaccessible for phosphatase action. Since T412 and T252 are established post translational events, and the kinases that phosphorylate these sites identified, the contention of their inaccessibility was certainly not plausible. The only other site that assumed significance in terms of its requirements for enzyme activity in this system, Ser394 believed to be co-translational, understandably continued to resist phosphatase action. Interestingly significant residual activity continues to be detected in the enzyme expressed in CHO-IR and NIH-3T3 cells even after the phosphorylation at T412 was more or less completely removed by phosphatase treatment lending credence to the observed resistance of BVr enzyme to phosphatase inactivation. Since the activation and rapamycin sensitivity of the enzyme has also been shown to critically depend on the recruitment of TOR kinase through amino and carboxy terminal TOR signalling (TOS) motifs it was imperative to examine, whether deletion of these motifs did indeed reproduce effects in accordance with the prevalent interpretations for mammalian cell system. S6K mutant truncated individually for amino and carboxy terminal TOS motifs showed characteristics no different than their wild type counterpart. Surprisingly the double mutant exhibited 2-3 fold more activity and partial resistance to rapamycin, in conformity with its reported behaviour in mammalian cells However, the explanation attributing this mutation to facilitate direct phosphorylation at the HM is completely redundant in view of its absence in the BVr enzyme. It is therefore, safe to conclude that TOR recruitment and the resultant phosphorylation at the HM does not mediate the inhibitory effects of rapamycin. Increased activity associated with the mutant may therefore, be simply due to steric freedom achieved by the truncated version of the enzyme ordinarily accomplished by activating phosphorylations. Rapamycin may instead potentiate a cellular event other than phosphorylation via binding or dissociation of a regulatory factor that changes the conformation of the enzyme such that it fails to engender these critical phosphorylations. Accordingly truncations that override such constraints would activate the enzyme and override rapamycin inhibition, without necessary dependence on phosphorylations, as stands reported for other such truncations.

In the absence of the activating phosphorylations and lesser activity associated with the BVr-enzyme, it can be concluded that the viral infection *per se* does not

activate the enzyme but instead appears to lock it in a state of activity comparable to a rather amplified basal (serum starved) state of the mammalian cells, in complete disagreement with the conclusion drawn by some investigators. The stimulus required to bring about activating phosphorylations may therefore, be either absent in insect cells or inactivated due to viral infection, undermining the existence of kinases responsible to bring about both AL and HM phosphorylation. It is therefore, obvious to contemplate that TOR signalling pathway that supposedly mediates both activation and rapamycin inhibition otherwise established in the insect cell system, fails to phosphorylate and activate the enzyme yet continues to mediate inhibition by rapamycin. Therefore, it seems that while the process of activation remains unaccomplished, viral infection preserves the events responsible for mediating rapamycin inhibition. In other words the activation and sensitivity to rapamycin appear to be two independent events, in complete contravention to prevalent hypotheses. Baculoviral infection in a way serves to provide a better system where the basal state, i.e. the form of the enzyme without phosphorylations at the HM and AL, is completely disengaged from the activation state otherwise quite difficult to achieve in a mammalian system. Since, it would be strange to contemplate a unique mechanism of rapamycin action for BVr-enzyme it is possible to contemplate that loss of activating phosphorylations in mammalian cells otherwise construed as mechanistic may in effect be only the consequence of rapamycin inhibition. It however, required further substantiation to validate the findings. Accordingly, further analysis with regard to functional analysis of TOR signalling and studying the sequence of occurrence of phosphorylations at critical residues (T412 and T252) was desired. Although both phosphorylations are equally important without a question however their individual roles in contributing to activation and mediating rapamycin sensitivity remains controversial. Phosphorylation of the enzyme at its HM brought about by rapamycin sensitive mTOR kinase is thought to prime the enzyme for PDPK1 dependent phosphorylations at AL, in accordance with the prevalent dogma of sequential phosphorylations. Accordingly inactivation of mTOR by Rapamycin resulting in loss of HM phosphorylation and resultant loss of phosphorylation at the AL is the model put forward to explain inhibition by rapamycin. The attribution of mTOR as the kinase responsible to engender HM phosphorylations, however is based

on trivial *in vitro* evidence carried forward simply on the basis of the ability of mTOR to bind rapamycin. Indirect evidences wherein deletion or modification of one or both TOR signalling motifs are shown to influence S6K 1 activity are also questionable for several reasons. Firstly, the enzyme deletion or modification of TOS motifs thought to be necessary for the recruitment of mTOR kinase continues to be phosphorylated at HM. Secondly, the redundance of TOR kinase mediated HM phosphorylation in mediating rapamycin inhibition of Bvr enzyme strongly suggest, that effects attributed to TOS motif deletion may simply be conformational and not necessarily attributable to the loss of TOR kinase input. Simultaneous loss of both HM and AL phosphorylations without any preference for the HM phosphorylation reported herein does not support the TOR kinase candidature either. Even if HM phosphorylation were to be lost first the relative loss of AL phosphorylation would be different both in time and quantum. In fact the data at time points as low as 1 min rapamycin exposure (not shown) did not show any deviation from the pattern in which the two bites de-phosphorylates. Although dramatic conclusions cannot be drawn at this point, it does extend a basis to revalidate the dogma of sequential phosphorylations. Incidentally, alternative model proposed recently by Keshwani *et al* while questioning the veracity of sequential phosphorylation model by suggesting AL to get phosphorylated prior to HM, fails to explain the loss of AL phosphorylation in response to rapamycin in the backdrop of established data implicating a rapamycin insensitive enzyme PDPK1 in phosphorylating this site both *in vitro* and *in vivo.*

We therefore support the contention that the two phosphorylations must in fact be co-ordinately regulated such that both phosphorylations are engendered concurrently during activation and lost simultaneously as a response to inhibition by rapamycin. We present systematic evidence that the two phosphorylations exhibit characteristics of coordinate turnover both in vivo and in vitro. Accordingly introduction of phospho mimicking mutations at the AL (T252E) or HM (T412E) in HEK 293 cells caused in vivo phosphorylations at T412 and T252 respectively. Although the extent of either phosphorylation ensured in the mutants was quite modest in comparison to WT, the indication was clear that the accordance of each phosphorylation was completely oblivious to the mutation at the other site to perhaps suggest that the phosphorylation

at one site was not an impediment for the phosphorylation at the other. In other words the two phosphorylations do not seen to follow a preferential sequence of occurrence, in contravention with the prevailing model. The lesser extent of phosphorylation in each mutant can however be attributed to the fact that glutamate in the background may incapacitate the enzyme to act as a good substrate for the kinase. In the back drop of data that T252E does not reproduce phospho mimicking due to steric sensitivity of the site, it can easily be argued that T412 phosphorylation in T252E mutant may occur on its own without any cue of phospho mimicking at the AL. Interestingly, resistance of T412 or for that matter T252 phosphorylation in the mutants, to inhibition by rapamycin is an indication that their turnover is certainly dictated by the background mutation in view of the fact that the two phosphorylation effectively are inhibited in the WT format, to suggest that each mutation did indeed contribute in the dynamics of the phosphorylation at the other site. Notwithstanding the counter argument, the cause associated *in vivo* occurrence of the phosphorylations was close resolve to cause and effect bias for the dynamics of these phosphorus in a baculoviral expression system wherein WT S6K, fails to engender AL and HM phosphorylations *in vivo*. Strikingly mutational intervention at either AL or HM resulted in corresponding phosphorylation at the much less the same way as was observed in HEK-293 cells. Since neither of the two phosphorylations occur in the WT enzyme, it is clear that they ensue as a result of the cue from the mutation at the other site, substantiating their interdependence and co-ordinate turnover. The effects associated with phospho deficient variants T252A and T452A closely resembling those of the phosphor mimicking variants, though paradoxical go to suggest that conformational state and not the negative charge *per se* may be responsible for their turnover dynamics, such that an active conformational state would result in coordinate phosphorylation at AL and HM and inhibitory conformational state propagated by rapamycin that may involve binding or release of an unknown factor or one of subunits of TOR-complex itself, result in their loss as a consequence (**Figure 3.15**). In other words presence of phosphorylation simultaneously at both sites would override the inhibitory conformation such that preventing the turnover at one site would lock the enzyme in fully active rapamycin resistant state. While it can be reproduced quite nicely in T412E mutant that mimics

T412 phosphorylation to a reasonable extent, it cannot unfortunately be reproduced in T252E situation, because it mimic phosphorylation even though it does bring in some conformational accordance to cause phosphorylation at T412. In the absence of T412 or T252 phosphorylation independently mediating inhibition by rapamycin, and continued inhibition of the enzyme by rapamycin, it can be concluded that the loss of phosphorylation associated with rapamycin cannot be mechanistic and has to be a consequence.

Discussion Part II

This part of the study aims to investigate whether the loss of HM and AL phosphorylations is a consequence of S6K1 inhibition specific to rapamycin or is associated with the inhibition of the enzyme in general. Two local anaesthetics (LAs) bupivacaine and lidocaine, whose mechanism of action is suggestive of their influence on signalling events other than TOR, were thus used as potential inhibitors of S6K1 to study whether the inhibition due to these drugs was associated with loss of HM and AL phosphorylations.

Local aesthetics (LAs) mediate growth inhibitory effects by influencing signalling molecules like p38 MAPK, Akt and ERK. Since growth regulatory and apoptotic mechanisms are largely interdependent and complexed further by cross talk between signalling molecules, the attribution of one or the other event to mediate such effects may be premature at this point. This is primarily because the influences of the other signalling pathways like S6K1 on cellular growth and apoptosis is as pronounced, if not more as the ERK or the other events influenced by LAs. In fact the effects associated with LAs that include metabolic stress, influence on nutrient homeostasis , apoptosis , inhibition of Akt etc directly or indirectly are suggestive of the contributory potential of this pathway in LAs mediated cellular damage. LAs induced inhibition of S6K1 reported herein is in accordance with the argument to put a lot of data into perspective. Inhibition of S6 kinase was associated with the loss of hydrophobic motif (HM) and activation loop (AL) phosphorylations at T412 and T252 respectively, attributed to inactivation of mTOR kinase that phosphorylate the enzyme at T412 or PDPK1- the downstream effector of PI3-kinase pathways that phosphorylates S6K at T252. In addition, several other kinases DRAK2, Akt, GSK3β including ERK are known to influence phosphorylation at the HM directly or indirectly. These molecules assume significance in the context of the observation that mTOR pathway fails to register its participation in S6K inhibition by LAs. Accordingly Akt inhibition by LAs , might explain the observed inhibition of S6K1, in view of its direct participation in S6K regulation, or indirect involvement through GSK3β . Similarly DRAK 2, a key player in cellular apoptosis known to influence S6K1 activation may constitute the other link inviting attention. The contribution of

direct ERK influence on S6K1 inhibition by LAs appears in context as much as other possibilities particularly in the light of the data describing MAPK-S6K co-inmunoprecipitation to establish cross talk between the two pathways, substantiated further by inhibition of RSK1 by bupivacaine reported herein. However inhibition of RSK1 by bupivacaine and not lidocaine though suggestive of bupivacaine being more potent than lidocaine , raised questions about its specificity for S6K1 inhibition. It however remains to be seen whether the time course of ERK inhibition overlaps with that of S6K1 or RSK1.The substrate specificities and other kinetic properties of RSK might suggest that its inhibition would represent extreme state of cellular stress than the one represented by S6K1 inhibition. Failure of lidocaine to inhibit RSK1 while supporting the potency argument may also indicate that its inhibition is only secondary to the inhibition of S6K1. Notwithstanding the participation of various phospho-dependent events in mediating the S6K1 inhibition by LAs, a mechanism independent of identified inputs deserves attention.

The inhibition of S6K1 activity by LAs has implications for their role in cellular proliferation especially in the context of their established anticancer properties. Accordingly studies delineating the mechanistic events leading to S6K inhibition by LAs may have relevance in clinical management of cancer and associated malignancies.

Conclusions:

- S6K enzyme expressed in insect cells is active towards phosphorylating S6, however activity of the enzyme is 4-6 folds lesser than the enzyme expressed in mammalian cells.

- The lesser activity associated with S6K immunoprecipitated from Sf9 cells is attributed to absence of phosphorylations at T412 (HM) and T252 (AL).

- Rapamycin inhibits S6K without any involvement of T412 and T252 phosphorylations.

- Baculovirally expressed S6K1 is resistant to phosphatase inactivation suggesting redundancy of phosphorylations in general and HM/AL phosphorylations in particular in mediating rapamycin inhibition.

- Phosphorylation at T412 is not dependent on TOR recruitment mediated through TOS signalling motifs.

- Activation or rapamycin sensitivity of the enzyme is regulated by coordinate dynamics of phosphorylations at HM (T412) and AL (T252), such that disappearance of one facilitates removal of the other phosphorylation to regulate rapamycin inhibition and opposite holds true for activation.

- Phosphorylation at T412 is regulated independent of rapamycin input both *in vivo* and *in vitro.*

- Rapamycin sensitivity and activation are two independent events

- Phosphorylations at the two residues (HM & AL) are mediated through binding or release of an unknown cellular factor, such that its binding or removal makes the two sites accessible for phosphorylation and *vice versa*

- mTOR independent pathways regulate S6 kinase indirectly to mediate several cellular processes like diabetes and aging.

- mTOR and PDPK1 phosphorylate S6K1 by a interdependent mechanism, such that phosphorylation at either site facilitates phosphorylation at the other site.

- Local anaesthetics induced growth inhibition is associated with inhibition of S6K activity in vivo which in turn prevents phosphorylation of S6 protein-a key protein involved in ribosomal biogenesis and synthesis of protein required for translation.

- Since inhibition of S6K by local anaesthetics is independent of any TOR input that hints at a possibility of cross talk with MAPK and Akt pathways which have already been shown to get inhibited by these drugs.

- Inhibition of S6K by LAs implies that this class of drugs has a potential to modulate several effector processes wherein S6K has been involved like cellular metastasis and apoptosis.

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